

ECOLOGICAL AND MOLECULAR STUDIES OF FUNGAL COMMUNITIES
ASSOCIATED WITH ROOTS OF *SALIX* SPP. GROWN UNDER HIGH DENSITY, SHORT
ROTATION INTENSIVE CULTURE

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By

Aura Helena Corredor

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ABSTRACT

In Canada, willow (*Salix* spp.) short rotation intensive cultures (SRIC) have been established to investigate their potential to produce biomass for bioenergy. Since root-associated fungal communities are involved in plant nutrition and disease susceptibility, it is relevant to understand their interactions with willows and their role on the sustainability of SRIC. In this project traditional methods together with molecular techniques were used to: *i*) assess the diversity of fungal communities in roots of willows and their relationship with major characteristics of SRIC *ii*) evaluate the effects on plant development of potentially beneficial and pathogenic fungi, and *iii*) identify arbuscular mycorrhizal fungal (AMF) species and their interaction with promising *Salix* clones.

Potentially pathogenic fungi were more dominant in diseased and recently planted cuttings than in healthy older ones. This suggested the occurrence of a positive shift prompted by the establishment of the cuttings on the site. *Gibberella/Fusarium* sp. and *Neonectria* sp. were the most dominant taxa particularly in diseased plants. Under greenhouse conditions the inoculation with potentially beneficial and potentially pathogenic fungi induced significant differences in root biomass but not in overall aerial biomass production. PCR-denaturing gradient gel electrophoresis was successfully standardized and used to identify arbuscular mycorrhizal fungal species associated with different clones. The results indicated a degree of specificity between AMF species and plant genotype.

Root-associated fungal communities appear to be useful to monitor the impacts of SRIC on soil ecology and their study may enlighten effective ways to increase the productivity of these biomass systems.

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1. INTRODUCTION

Short rotation intensive culture (SRIC) of willows (*Salix* spp.) offer many advantages for biomass production (Volk *et al.* 2004); however, several factors influencing these systems need to be considered before this technology can be promoted as a safe and profitable alternative for Canadian farmers.

Root-associated fungal communities influence plant nutrition, disease susceptibility and environmental adaptability (Sylvia 2005). Based on their effects on plant development, root-associated fungi can be classified as beneficial, pathogenic and neutral. Beneficial groups are usually related with improvement of plant nutrition, resistance to environmental stress and defense mechanisms against diseases. On the other hand, pathogenic fungi are associated with disease outbreaks and must be carefully monitored and managed to prevent their dispersion. Willow species have been reported to associate with a number of fungal functional groups in natural forests but little is known about these associations in SRIC systems (Dhillon 1994; Hubbes 1983; Paradi and Baar 2006).

Biomass plantations may differ substantially from the environments in which *Salix* spp. are naturally found. SRIC presuppose clonal propagation, coppice and management practices to maximize productivity (Labrecque and Teodorescu 2005; Smaliukas and Noreika 2005). These conditions increase the susceptibility of willows to disease development. Furthermore, the use of marginal and cleared arable land is usually challenging for the establishment of biomass plantations (Khasa *et al.* 2002). Intensive soil transformation, caused by farming management practices such as tillage and fertilizers and herbicides application, may be partially responsible for these difficulties. Fungal communities that play important roles in willow nutrition and disease protection may be reduced as a result of agricultural activities (Helgason *et al.* 1998) while infestation by pathogenic fungi is not unusual in soils that have undergone cultivation.

It is in the early stages when the majority of plant losses are typically reported for SRIC systems (Volk *et al.* 2004); therefore, understanding fungal–plant interactions during that period of time may be fundamental to implement strategies that contribute to cutting survival. Surveying the diversity of fungal communities associated with healthy and diseased plants is the first step in identifying beneficial and pathogenic groups in roots of willow plantations.

Among the beneficial fungi associated with roots of *Salix* spp., mycorrhizas are perhaps the most extensively studied (Hryniewicz *et al.* 2009; Paradi and Baar 2006; Rooney *et al.* 2009; Van der Heijden *et al.* 1999; Van der Heijden and Sanders 2002). Species belonging to this group are involved in plant nutrition and heavy-metal mobilization and uptake in willow stands (Baum *et al.* 2006; Van der Heijden and Kuyper 2001b). Nonetheless, important questions regarding their diversity and interactions with willows grown under SRIC have not been substantially explored due in part to the lack of accurate methodologies. Their importance, however, is widely recognized and attempts have been made to integrate them in the development of biomass plantations (Quoreshi *et al.* 2008).

In comparison to mycorrhizal communities, little is known about non-mycorrhizal fungi and their role in cutting development. The relevance of these fungi has been only recently demonstrated for specific cases. The presence of particular species of saprotrophic microfungi in the rhizosphere of *S. viminalis* and *S. x dasyclados* was associated with changes in the activity of enzymes involved in nutrient release (Baum and Hryniewicz 2006). Root-associated fungi have also been correlated with improved tolerance of *S. caprea* to soil contaminated with lead and cadmium (Likar and Regvar 2009). Additionally, greenhouse tests indicated that the inoculation with non-mycorrhizal fungi significantly enhances plant growth and development of *S. fragilis* in both contaminated and non-contaminated soils (Adams *et al.* 2007). This evidence suggests that an improved understanding of the interaction between willows and their non-mycorrhizal root-associated fungal communities could result in the development of novel approaches to increase biomass production and adaptability to harsh environments.

Fungal pathogens have been important threats for the success of willow biomass plantations. Leaves and stem are highly susceptible to a number of rusts and cankers caused principally by species and hybrids of *Melampsora* and *Marssonina* (Hunter *et al.* 1996; Pei *et al.* 1996; Royle and Ostry 1995; Spiers 1998). Root diseases, on the contrary, have not been of main concern so far. Diseases may appear after introduction by natural or anthropogenic means or they may result as a consequence of a breakdown in host susceptibility (Royle and Ostry 1995). Therefore, strong pathogenic species remaining in cleared agricultural land may be a potentially important factor threatening the survival of young cuttings.

The interactions with root-associated fungal communities are determined by the genetic background and specific characteristics of each willow clone (Baum *et al.* 2006; Baum *et al.*

2002; Kahle *et al.* 2005). Enhanced biomass productivity is the direct result of physiological traits expressed by a genotype but can also be an indirect consequence of the improved activity of its root-associated fungal communities. Selecting suitable clones for biomass production may, therefore, take into consideration their ability to coexist with and profit from fungal species living in their roots. Moreover, monitoring the responsiveness of root-associated fungal communities may be a valuable resource to evaluate the effects of SRIC systems on soil ecology (Baum *et al.* 2009; Kahle *et al.* 2005).

Efficient approaches must be adapted to monitor fungal communities associated with the roots of willows grown under SRIC. Molecular techniques have been favored for ecological studies of environmental samples due to their efficiency and accuracy (Anderson and Cairney 2004). Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting technique that has been successfully used for the study of fungal communities in diverse environments (Muyzer *et al.* 1993; Muyzer and Smalla 1998). Changes of endophytic communities associated with the roots of *Salix* have been studied using similar fingerprinting techniques and significant correlations between environmental characteristics and fungal diversity have been detected (Likar and Regvar 2009). In the case of SRIC, communities of arbuscular mycorrhizal fungi (AMF) could be important candidates to be studied with these techniques given the difficulties for their isolation and identification (Liang *et al.* 2008; Ma *et al.* 2005).

The studies embodied in this project were proposed to evaluate:

1. The influence of plant health status, clone, plantation age and province on the root-associated fungal communities of promising *Salix* genotypes grown under SRIC in the Canadian Prairies.
2. The effects of the inoculation of potentially pathogenic and potentially beneficial fungi on the development of *Salix* cuttings.
3. The differences or similarities between *Salix* genotypes in regard to their associated AMF communities.

The document starts with a review of major aspects of biomass plantations, root-associated fungal functional groups in *Salix* spp. and fundamental aspects and applications of DGGE for fungal ecology studies. Chapter 3 explores the diversity of root-associated fungal

communities and the effects of major characteristics of SRIC on the composition of those communities. Chapter 4 evaluates the effects on the growth and development of willow cuttings caused by the inoculation of potentially beneficial and potentially pathogenic fungi isolated from the roots of *Salix* spp. Finally, Chapter 5 focuses on the standardization and application of DGGE to identify and compare AMF communities associated with different *Salix* clones.

2. LITERATURE REVIEW

2.1. Willow Crops for Biomass Production

Traditional uses of willows (*Salix* spp.) can be tracked back into ancient human societies; however, it is only in this century when the need for environmentally-engaged alternatives to energy production and contamination problems has revealed an enormous potential for the use of these plants (Ball *et al.* 2005). Traditional uses of willows range from primary material for baskets, arrow shafts, fish traps, furniture, pulp and paper to more sophisticated applications in Eurasia and North America where an analgesic (salicin) was obtained from the bark of the trees (Kuzovkina and Quigley 2005). Willows possess biological traits that make them exceptionally useful. They are able to grow through a wide variety of climates and soils (Ball *et al.* 2005) and different species exist in all continents (Argus 2007). So far, 450 species have been described, most of them native of temperate zones and some others native of subtropical areas (Argus 2007).

The high biological plasticity of willows has been essential to discover and develop innovative uses, in accordance with human needs and concerns (Christersson 2008). The benefits obtained from willows are not restricted to wood derived products but also include non-wood products and services (Ball *et al.* 2005). In recent years, for example, willows have been adopted as the main components of environmental projects involved in phytoremediation (Quaggiotti *et al.* 2007), land reclamation, improvement of diminished ecosystems and several other applications that take advantage of the numerous physiological and ecological properties of these species (Ball *et al.* 2005). Characteristics such as the ability to grow in sites of low nutrient availability, numerous seed production, vegetative propagation and tolerance to high sunlight exposure, make willows a very suitable resource for environmental purposes (Smart *et al.* 2005).

Aware of the importance of implementing alternative energy resources, countries including Canada, the United States and Sweden have shown special interest in the use of willows for production of biomass (Dimitriou and Aronsson 2005; Wright 2006). In temperate regions this application would have a remarkable impact since bioenergy produced from *Salix* spp. would be an environmentally-friendly replacement to fossil fuels (Dimitriou and Aronsson 2005). Bioenergy derived from willow biomass appears to have several advantages when compared to energy derived from traditional fuels. According to Volk *et al.* (2004) during the

process of biomass production there is no net flux of carbon (in the form of CO₂) to the atmosphere because the amount that plants accumulate to grow is the same amount that is released when the biomass is transformed into energy. This and other benefits associated with bioenergy derived from willows (Volk *et al.* 2004) make it suitable to partially remedy the greenhouse gases accumulation and consequent climate change which currently constitute one of the main environmental concerns of the global community.

Across Canada an ambitious research project to investigate the implementation of short rotation intensive crops (SRIC) of willows and poplars (*Populus* spp.), with primary focus on bioenergy generation, is being developed (Labrecque and Teodorescu 2008). The project intends to study relevant aspects of the technology of biomass production before stimulating farmers to initiate their own plantations. In order to achieve this goal, several sites have been established in different provinces including Ontario, Manitoba, Saskatchewan and Alberta. The sites have been established at different times during the last decade and have been planted with different *Salix* clones. Some of the clones that are being evaluated include Hotel (*S. purpurea* L.), Charlie (probably *Salix alba* x *glatfelteri*), Juliet (*S. eriocephala* Michx.), India (*S. dasyclados* Wimm.), SX64 (*S. miyabeana* Seemen), SX61 (*S. sachalinensis* Fr. Schm.), and SV1 (*S. dasyclados*), among others. From this project, Labrecque and Teodorescu (2003) working with plantations of *S. viminalis* L., reported the highest woody production ever achieved in Canada. This and other results obtained so far seem very positive for the development of the technology for biomass production. However, further research is needed not only on the technology itself but also on the many different biological and environmental aspects related to SRIC plantations.

In fact, as originally recognized by Abrahamson *et al.* (1998), the global adoption and acceptance of bioenergy produced from willows biomass will be highly influenced by a careful assessment of its sustainability at the ecological and socio-economical levels. The importance of this matter is reinforced by the fact that in 2005, plantations together with agroforestry systems and trees outside forests, represented up to 6% of the total willow area in the world (Ball *et al.* 2005). Based on reports from the International Poplar Commission, it was concluded that the tendency was to increment the use and cultivation of willow (Ball *et al.* 2005). The first formal attempt to evaluate the sustainability of willow SRIC in North America was made by Volk and collaborators (2004). While the conclusions of this report were very promising, the authors

stressed the need for developing additional reliable indicators to assess the benefits and to support the appropriateness of these crop systems.

2.2. Fungal Functional Groups Associated with Roots of *Salix* spp.

Understanding soil properties is determinant for the improvement of agricultural and forestry systems (Howard 2006). Fertilization and irrigation programs are common practices intended to effectively compensate for soil deficiencies affecting crop yields (Howard 2006). Nevertheless, soil quality not only depends on its physical and chemical characteristics but it is also influenced by biotic processes that take place in it (Van Bruggen and Semenov 2000). Thousands of organisms are constantly interacting with the edaphic environment, simultaneously modifying and being modified by the surrounding conditions. Soils are complex ecosystems where vital processes for the global dynamics are supported by strong connections among their components (Doran and Zeiss 2000).

The relationships between soil fungi and plant development have been the interest of numerous studies for agricultural and ecological purposes. Fungal communities are major components of the soil biota and together with bacteria are involved in the cycling of elements (Anderson and Cairney 2004). In fact, fungal metabolic processes such as mineralization of nitrogen and solubilization of phosphorus make these nutrients available to plants (Sylvia 2005); nutrients that otherwise would remain practically inaccessible. Furthermore, it has been demonstrated that specific fungal communities belowground, i.e. mycorrhizas, can influence the species composition of plant communities aboveground (Van der Heijden and Sanders 2002). In other words, the functioning of the communities above and belowground is strongly determined by their permanent interaction (Molina *et al.* 1992). As a result, ecological pressures such as that of agriculture and forestry systems are expected to directly affect the stability of soil fungal communities, especially on those that are in close contact with the root system (Helgason *et al.* 1998; Van Bruggen and Semenov 2000).

Root-associated fungal communities are studied using different yet complementary approaches (Anderson and Cairney 2004). Better understanding is gained when traditional studies, formulated in terms of species richness (biodiversity), are complemented with the study of the functional diversity of such species (Van Bruggen and Semenov 2000). From an ecological perspective, root-associated fungi can be classified into functional groups according to

their life form and/or substrate utilization. For example, Gebauer and Taylor (1999) distinguished four different fungal functional groups resulting from the combination of two life forms (ectomycorrhizal fungi and saprophytes) and two types of nitrogen source (organic and inorganic). Another example is the classification by Mulder *et al.* (2003) by which four different functional groups were recognized, i.e. mycorrhizal fungi, fungi on decayed or buried wood, epiphytic fungi on herbaceous tissues and saprophytic dung fungi. Another method that has been proposed to study fungal functional diversity is the use of microtiter plates containing different sources of carbon (Dobranic and Zak 1999) or nitrogen (Grizzle and Zak 2006).

Based on their effects on plant performance, root-associated fungi in agricultural systems can be broadly classified into three major functional groups: pathogenic, beneficial and neutral. The dominance of one specific functional group over the others is stimulated or not by the predominant conditions (Mulder *et al.* 2003) and their ability to use the available resources. Pathogens infect plants and, depending on their mode of action, a variety of symptoms can develop in the host (Agrios 2005). In the majority of cases, these infections interfere with the optimal development of the plants and propagate rapidly within a crop system. On the other hand, beneficial fungi may also infect the roots (endophytes) but no negative effects are observed. Moreover, the presence of these fungi offers benefits for the development of the host plant. Such benefits may include an increase in nutrient absorption, protection against pathogens and resistance to extreme environmental conditions.

Little is known about the actual effects of willow SRIC systems on root-associated fungi despite the role of these microorganisms in plant physiology and ecology (Van der Heijden 2001) or their potential as indicators of soil quality (Van Bruggen and Semenov 2000). Research done to date has focused mainly on one particular group of beneficial fungi (Baum *et al.* 2002; Puttsepp *et al.* 2004) but it is known that numerous fungal communities coexist in close relationship with the roots of willows (Dhillion 1994; Vujanovic and Labrecque 2002, 2008). For example, it has been reported the ability of willows to form dual associations with endo and ectomycorrhizal fungi (Van der Heijden 2001), as well as the presence of several decay fungi some of which can cause root diseases. Several studies have identified also a group of endophytes commonly denominated dark septate endophytic fungi (Trowbridge and Jumpponen 2004), whose function is still not clear.

The influence of each of these groups of fungi on the plants might affect the success of the plantation and biomass yield even during the first stages of crop establishment, which suggests that special attention should be given to each of them.

2.2.1. Fungal Pathogens

The awareness of potential deleterious agents is fundamental for the adoption of management policies in any given crop. Willows are susceptible to a range of pathogens which can be grouped according to the plant organ that they mainly affect (Vujanovic and Labrecque 2008). Pests and pathogens of willows are commonly found in the aerial tissues of the plants and tend to be highly destructive. Hubbes (1983) identified potential diseases of bioenergy crops, calling special attention to those occurring in leaves and stems. Furthermore, Royle and Ostry (1995) and later Pei *et al.* (1996) studied the ecology, genetics and epidemiology of rusts in willow plantations, with the objective of developing a strategy for the control of the causal pathogens. *Melampsora* sp. is a harmful fungal genus commonly found in willows and covers numerous species causing rust diseases (Spiers 1998). Additional genera causing serious foliar damage in *Salix* spp. are *Marssonina* and *Fusicladium* (Royle and Ostry 1995; Vujanovic and Labrecque 2002, 2008). Regarding stem diseases several species causing cankers have been isolated. These include, among others, *Botryosphaeria ribis* Grossenb. & Duggar, *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk and many species of *Valsa* (Hubbes 1983; Vujanovic and Labrecque 2002).

Willow plantations may be prone to diseases given their clonal nature. A model proposed by Hunter *et al.* (1996) to study the development of diseases in plantations of the United Kingdom, indicated significant statistical interactions between disease development and clones, as well as between geographical regions and seasons of the plantations. Later, Vujanovic and Labrecque (2002) completed an inventory of pathogenic agents that could be found in *Salix* spp. bioenergy plantations in Eastern Canada. The list included coelomycetous species and *Coniothyrium* sp. among the pathogens isolated from roots. More recently, Vujanovic and Labrecque (2008) studied the community of Ascomycota fungi that could be found either as pathogens or biocontrol agents in green wall structures of *S. viminalis* in Quebec. Remarkably, the study did not find any infection by *Melampsora* sp. which previously had been identified as an important threat for plantations in Europe and New Zealand (Hunter *et al.* 1996; Spiers 1998).

Instead, some of the most frequent genera isolated from diseased plants were *Venturia*, *Cryptodiaporthe*, *Drepanopeziza* and *Glomerella*.

In contrast to the importance of leaves and stem diseases, decay and root rot are not considered significant problems in *Salix* spp. Some species with potential to attack roots of decayed willows include *Armillaria mellea* (Vahl) P. Kumm., *Chondrostereum purpureum* (Pers.) Pouzar, *Phymatotrichum omnivorum* Duggar, and some species of the genera *Polyporus* and *Trametes* (Hubbes 1983). Nonetheless, most of these species are common saprophytes and very few have been considered as a serious risk for living trees.

2.2.2. Mycorrhizas

Mycorrhizas are found in the majority of terrestrial plants and are generally associated with benefits for plant performance (Van der Heijden and Sanders 2002). Arbuscular mycorrhizas and ectomycorrhizas are the most widespread types of mycorrhizas. The coexistence of these two types in the same root system has been reported for some plants, including a number of *Salix* species.

The role of each type of mycorrhiza in the development of willow is variable in time and magnitude (Van der Heijden 2001). Van der Heijden (2001b) observed that the beneficial effects of arbuscular mycorrhizal fungi (AMF) were evident during the first twelve weeks of *S. repens* cuttings. On the contrary, the benefits of ectomycorrhizal fungi (EMF) occurred during the late part of the growth season, when the cuttings had developed extensive root systems. According to Van der Heijden (2001) the differential benefits obtained from each type of mycorrhizal fungi could be related with the physiological status of the plants and their differential nutritional requirements at each growth stage.

In addition to the growth stage of the host plant other factors may be considered to influence the development of mycorrhizas in roots of willows. Studying the role of moisture, Lodge (1989) found that the highest colonization by EMF in *Salix* spp. occurred in transects where the soil was both well drained and very moist, whereas in dry soils AMF colonization dominated over that of EMF. Nutrient availability plays also an important role in determining the development of mycorrhizas (Sasaki *et al.* 2001). In fact, EMF diversity changes according to the content of inorganic nitrogen (Lilleskov *et al.* 2002) and AMF colonization tends to decrease when the host plant is growing in soils with high phosphorus content (Smith and Gianinazzi-

Pearson 1990). Thus, the dynamics of mycorrhizal communities associated with roots of *Salix* spp. appears to be very variable and dependent upon the specific conditions under which the plants are grown.

Several researchers have attempted to assess the species diversity of these two mycorrhizal types in natural ecosystems where *Salix* species are main components of the plant community (Beauchamp *et al.* 2006; Dhillion 1994; Paradi and Baar 2006; Trowbridge and Jumpponen 2004; Van der Heijden *et al.* 1999). From these studies two conclusions can be drawn: first, even though *Salix* spp. are able to form both kinds of mycorrhizas, some species present only a very low or even no colonization by AMF (Paradi and Baar 2006). Second, when AMF are present, they are more active in young cuttings whereas EMF appear to be the most active mycorrhizal type in mature individuals (Van der Heijden 2001). These observations may be the result of different nutritional requirements characteristic of different plant growth stages or the result of the environmental conditions where the plants are grown.

In a comparative study of EMF diversity above and belowground, in the Dutch Wadden Isle of Terschelling, Van der Heijden (1999) found three genera, i.e. *Lactarius*, *Russula* and *Xerocomus* to be site specific whereas *Cortinarius* sp., *Hebeloma* sp. and *Paxillus* sp. were considered as being generalists. Previously, Dhillion (1994) had identified *Cenococcum* as the most abundant ectomycorrhizal genus and *Glomus tenuis* as the AMF colonizing the roots. In other work, Paradi and Baar (2006) described the mycorrhizal diversity in white willow (*S. alba*) forests of different age. In that study the most abundant ectomycorrhizal genera were *Tuber*, *Hebeloma*, and a non identified telephoroid species while AMF colonization was not detectable. On the contrary, Beauchamp *et al.* (2006) found a considerable richness of AMF species in stands of *Populus-Salix* along the Verde River in Arizona. The authors recovered spores from *Glomus* sp., *Acaulospora* sp., *Entrophospora* sp. and *Archaeospora* sp. and found a relation between the AMF diversity and the age of the stand, the distance to the channel of water, the soil moisture and the vegetation cover.

Since most of the studies on mycorrhizal diversity of willows have been done in natural forests, only limited information is available about the actual conditions in bioenergy plantations. Puttsepp *et al.* (2004) worked with plantations of *S. viminalis* and *S. dasyclados* clones where the most common EMF were *Hebeloma* sp., *Laccaria* sp., *Phialophora finlandica* C.J.K. Wang & H.E. Wilcox, and species from the Telephoraceae family. Regarding AMF, the information is

even scarcer in reference to the species that can be found colonizing the roots. There are, however, studies on the effects that *Salix* spp. afforestation and the use of nitrogen fertilization have on the AMF spore density and mycorrhiza formation (Baum *et al.* 2002; Kahle *et al.* 2005). There are also indications that AMF colonization may differ between clones grown in the same site (Khasa *et al.* 2002), which would suggest different degrees of susceptibility and probably differential benefits for each clone.

2.2.3. Other Beneficial Endophytes

In addition to mycorrhizas, other root-associated endophytes can be beneficial for willow. A number of saprotrophic species have been detected in the roots of willows in natural environments as well as in biomass plantations (Becerra *et al.* 2009; Chatli *et al.* 2008; Dhillon 1994). The enzymatic activities of these fungi are essential to make nutrients available for plants, although the resultant beneficial effects may be substantially different depending on the *Salix* species. Baum and Hryniewicz (2006) found that the acid-phosphatase and protease activities were significantly different between clonal plants of *S. viminalis* and *S. x dasyclados* grown under similar conditions. These two species of *Salix* present differences in the association that they establish with saprotrophic fungi, which may explain the differences in terms of nutrient mobilization observed for both clones (Baum and Hryniewicz 2006). Another way that saprotrophs could benefit *Salix* spp. is by positively affecting the activity of mycorrhizas through the solubilization of phosphorus and/or nitrogen (Babana and Antoun 2006) or by acting as biocontrol agents preventing the development of diseases.

Dominating saprotrophic genera in the rhizosphere and litter of *Salix* spp. plantations include *Cladosporium*, *Fusarium*, *Mortierella*, and *Cylindrocarpon*, among others (Baum and Hryniewicz 2006; Hubbes 1983; Likar and Regvar 2009). All of these genera can be found also in soils of less disturbed sites such as boreal forests.

In addition to the common saprotrophs, fungi recognized among the group of dark septate endophytes have been also repeatedly identified in roots of *Salix* spp. (Becerra *et al.* 2009; Fernando and Currah 1996; Likar and Regvar 2009). This group may be linked to a nutritional role in plants as well as to other ecological functions such as the utilization of organic material, the alteration of the host water uptake and the host drought and heat tolerance (Mandyam and Jumpponen 2005). Until 1998 a total of 16 species of Salicaceae had been reported to be

colonized by dark septate endophytes (Jumpponen and Trappe 1998). In willows growing in a natural ecosystem, Trowbridge and Jumpponen (2004) observed that almost 26% of the root length was colonized by these fungi, a percentage that exceeded that of mycorrhizal fungi. Species of this group which are able to colonize *Salix* spp. include *Leptodontidium orchidicola*, *Phialocephala fortinii*, *Cadophora* sp. and *Phialophora* sp. (Fernando and Currah 1996; Likar and Regvar 2009). These fungi have been found to be able to colonize the roots of *S. glauca* L. and *S. caprea* L. and to develop characteristic structures denominated microsclerotia.

The presence of beneficial root-associated fungi may cause a direct increase of plant biomass. For example, the inoculation with *Trichoderma harzianum* Rifai 1295-22 increased the growth rate and development of cuttings of *S. fragilis* L. significantly more than the inoculation with the ectomycorrhizal fungi *Pisolithus tinctorius* (Adams *et al.* 2007). In addition to the biomass, other factors that have not been studied but could be affected by the presence of beneficial endophytes may include changes in the photosynthetic activity of the plants, the increment of the reproduction capacity or the accumulation of certain elements in the tissues. Little is known of the benefits that endophytes other than mycorrhizas may cause in *Salix* spp. plants and, therefore, it could be an area worthy of investigation for the development of bioenergy plantations.

2.3. Denaturing Gradient Gel Electrophoresis (DGGE) in Fungal Studies

The size and complexity of root associated fungal communities present a challenge for their study and understanding (Bridge and Spooner 2001). Traditionally, researchers have used culture-dependent approaches to draw conclusions about these communities and the relationships existing within and between them (Bridge and Spooner 2001). However, it is widely accepted that such approaches generally underestimate the real composition of the fungal communities both in taxonomical and functional diversity due to the bias towards species that are able to effectively grow in artificial conditions (Zak and Visser 1996).

The advancement in fungal ecology, as well as many other research areas, has considerably benefited from the implementation of molecular techniques (Anderson and Cairney 2004). The most popular technique, polymerase chain reaction (PCR), is based on the examination of nucleic acids and is characterized for its simplicity and suitability to specifically target populations of interest (Atkins and Clark 2004). This tool has been used for the

development of more complex techniques, including denaturing gradient gel electrophoresis (PCR-DGGE), which go beyond the mere amplification of microbial DNA to perform comprehensive ecological studies (Anderson and Cairney 2004; Muyzer and Smalla 1998; Van Elsas *et al.* 2000).

PCR-DGGE was initially used for medical purposes and subsequently was adopted for microbial ecology studies (Muyzer and Smalla 1998). After its first introduction, the technique proved to be a powerful tool for such studies due to the possibility of working directly with environmental samples without a previous isolation of the microorganisms. In addition, the technique offers the possibility of comparing multiple samples at once thus allowing the observation of spatiotemporal changes (Anderson and Cairney 2004). Its extensive use during the last decades has increased the understanding of the ecology of different microenvironments (Muyzer and Smalla 1998).

A complete and detailed review of the theoretical criteria and practical application of this technique can be found in the document by Muyzer *et al.* (1998). Briefly, DGGE is grounded on the basis of the higher stability of the pair guanine-cytosine (G-C) compared to that of the pair adenine-thymine (A-T). Nakatsu (2007) summarized the procedure of a typical DGGE in the following steps: first, samples are collected and total DNA is extracted, then it is amplified by PCR targeting a specific sequence. The targeted sequence has to be specifically selected to suit the objectives of the study; however, the size of the fragments may not exceed 500 base pair (bp) since bigger fragments may not be appropriate for the resolution capacity of the gel.

The resultant amplicons are then run in a gradient gel electrophoresis which is created by using increasing concentrations of urea and formamide. When the DNA comes in contact with these chemicals it denatures, losing its double-strand conformation with the consequent slow down of its migration in the gel (Muyzer *et al.* 1993). A sequence with high content of G-C would be harder to denature than a sequence rich in A-T. In fact, the G-C rich sequence requires higher concentrations of the denaturing agents before it totally stops migrating (Muyzer *et al.* 1993). Consequently, although the PCR products amplified from different species might have the same size, they are likely to have different composition of nucleotides, thus generating a different banding pattern in the gel (Kisand and Wikner 2003).

The process ends when the bands in the gel are revealed using one of several different available protocols, from which ethidium bromide is the most common (Muyzer *et al.* 1993). In

theory, the banding pattern obtained would reflect the actual composition of the sample studied; however, only species representing more than 1% of the total population would be visible in the gel (Muyzer and Smalla 1998). Additionally, the intensity of the bands can be interpreted as the relative abundance of the species in the population, and new software packages have been developed to perform statistical analyses to draw ecological conclusions using the information provided by the banding pattern (Fromin *et al.* 2002).

Some disadvantages of the PCR-DGGE include the presence of multiple bands for a unique species which can lead to an overestimation of the real diversity (Kisand and Wikner 2003). Generally, it is recommended to excise problematic bands from the gel and sequence them, thus avoiding inaccurate interpretations. Another disadvantage is related to the first part of the procedure, where the PCR amplification can be biased due to the characteristics of the primers. It is a problem frequently reported (Anderson and Cairney 2004) which causes the preferential amplification of only certain templates. This results in increased intensity of those bands in the gel despite the original relative abundance of the template in the initial sample. To overcome such a difficulty, numerous authors have designed highly specific primers (Gardes and Bruns 1993; Gollotte *et al.* 2004; Vainio and Hantula 2000; White *et al.* 1990). Most of these primers have been designed to target either of the regions of the RNA gene due to the suitability of these sequences for interspecific differentiation (Anderson and Cairney 2004). Nonetheless, results from different studies are contradictory regarding the specificity of those primers for particular groups (Anderson and Cairney 2004; Borneman and Hartin 2000).

DGGE has been a useful tool for the study of soil fungal communities in different crops, despite its technical disadvantages. For instance, Yergeau *et al.* (2005) used DGGE to study *Fusarium* sp. in asparagus crops, and later, it was employed for the study of *Fusarium* sp. together with AMF (Yergeau *et al.* 2006) as well as for the study of both *Fusarium* sp. and *Pyrenophora* sp. in durum wheat (Mavragani 2008) and the study of AMF communities in agricultural soils (Ma *et al.* 2005). In those works, the use of nested PCR coupled with the design of specific primers, allowed the comparison of the presence of *Fusarium* sp. in two different plant health statuses, the assessment of the biodiversity of particular fungal groups and the evaluation of the impact of preceding cultures on the prevalence of specific fungi in current crops. Furthermore, in a recent study (Likar and Regvar 2009), temperature gradient gel electrophoresis (TGGE), a technique closely related to DGGE, was used to study endophytic

fungus communities living in the roots of *S. caprea* L. growing in a metal polluted site. The authors found this technique to provide a wide coverage of the fungal species in the major phyla, Ascomycota and Basidiomycota, some of which cannot be easily obtained or identified in culture. These technique may, therefore, be useful to facilitate the study of the dynamics of relevant fungal groups in the roots of willows grown under SRIC thus contributing to the understanding and improvement of these biomass production systems in Canada.

3. FUNGAL DIVERSITY ASSOCIATED WITH ROOTS OF WILLOWS GROWN UNDER SHORT ROTATION INTENSIVE CULTURE

3.1. Abstract

Fungal communities associated with the roots of willows grown under short rotation intensive culture (SRIC) were isolated in order to evaluate their relationship with plant health status, clone, province and year of establishment. The communities were highly dominated by fungi belonging to Ascomycota and only a few Zygomycota and Basidiomycota were detected. The results suggested a shift from communities dominated by potentially pathogenic fungi during the first year of plantation establishment to communities with an increased presence of potentially beneficial fungi during the second year. Among the potentially pathogenic fungi, *Gibberella/Fusarium* and *Neonectria* taxa appeared to be the most important threats. On the other hand, *Alternaria*, *Penicillium* and *Phomopsis* taxa were identified as potentially beneficial fungi given their dominance in roots of healthy plants. The composition of the fungal communities appeared to be associated also with clone identity. These results support the importance of including root-associated fungal communities as an integral part of the development of the SRIC in order to control potentially pathogenic fungi and/or promote potentially beneficial species.

3.2. Introduction

Energy derived from biomass is expected to decrease, at least in part, the dependence of modern societies on fossil fuels. Woody crops are predicted to become the main source of biomass during the coming years (Volk *et al.* 2004); therefore, their development is becoming a critical component of the strategy to reduce the current amounts of CO₂ emissions in several developed countries.

Short rotation intensive cultures (SRIC) of willows (*Salix* spp.) are particularly attractive to produce biomass due to numerous economical, ecological and social advantages associated with these systems (Abrahamson *et al.* 1998). The first willow SRIC for biomass purposes were established during the past decades in Europe and the United States (Volk *et al.* 2004). The aim of these crops has been to investigate their feasibility to produce substantial amounts of biomass in a sustainable way. Experience has been gained and an increasing amount of information has

been generated regarding the functioning and management of these systems (Volk *et al.* 2006). Although the results of most of the studies carried out in those crops support the implementation of SRIC in a larger scale; numerous aspects remain to be considered before the establishment of *Salix* spp. plantations can be promoted as safe and profitable for farmers.

Following the successful experiences in Europe and the United States, Canada started its own research program on willow crops. SRIC were recently established across the country to evaluate the performance of these systems under the Canadian conditions. In agreement with the requirements of the project, plantations were established on areas designated as agriculture land. Such areas are vastly confined to the Prairie provinces, namely Alberta, Saskatchewan and Manitoba, which are reported to hold 80% of Canada's farmland (Statistics Canada. Agriculture Division. 2004).

Although the Prairies include extensive areas for cultivation, the predominant climatic conditions in these regions are extreme and may present a challenge for the establishment of SRIC. Moreover, the development of SRIC in cleared arable land could be arduous given the intensive site transformation resultant from the previous agricultural activities (Khasa *et al.* 2002). Being typical pioneer species, willows possess biological mechanisms to thrive under sub-optimal nutritional conditions (Kuzovkina and Quigley 2005) but in SRIC systems numerous factors can further limit plant performance.

It is generally projected that the majority of plant losses in a SRIC occur during the first years after planting (Sage 1999). This could be a normal consequence of the transplant shock or competition with weeds; nonetheless, this situation could be influenced also by the interaction with fungal communities remaining from previous crops or already existent in the cuttings. In particular, root-associated fungi can be highly involved with the nutrition and protection of young plants (Baum and Hryniewicz 2006).

A considerable amount of information exists on the fungal communities found in association with the roots of willows in natural ecosystems but little is known about those present in SRIC (Baum and Hryniewicz 2006; Muhlmann and Peintner 2008; Paradi and Baar 2006; Puttsepp *et al.* 2004). The fungal communities that are first encountered by the developing root systems could be greatly dominated by species with the potential to induce diseases (Hubbes 1983). Such species would then be prompted to infect the cuttings given the susceptibility characteristic of this growth stage. In addition, the introduction of willows in arable land could

carry another risk. Willows can be susceptible to non-specific pathogens present on-site since in arable systems the levels of dominance of potentially deleterious agents could be considerable (Van Elsas 2002) and even higher than the levels found in natural forests.

Contrary to potentially pathogenic fungi, some integrants of the root-associated fungal communities may be important contributors to the establishment of *Salix* spp. Previous studies have found that willows grown in semi-natural conditions can be associated with potentially beneficial and biocontrol fungal species (Vujanovic and Labrecque 2008). Therefore, species of these functional groups may be also expected to exist as part of the root-associated fungal communities in willow plantations. Their benefits in SRIC could range from increasing plant nutrition to actively contribute to the improvement of plant resistance against stressful abiotic factors. Moreover, root-associated biocontrol species could be potentially considered for an integrated pest management program in SRIC since they are likely to be adapted to the predominant environmental conditions in these systems.

In addition to the appropriate management of the natural resources available in-site, the development of SRIC also involves the selection and breeding of suitable plant genotypes to achieve optimal production levels (Smart *et al.* 2005). In the case of the Prairies, the ideal clonal varieties must exhibit a good degree of adaptation to the extreme climatic conditions of the region, and be highly resistant to disease development. These parameters can be evaluated and better understood through the study of the differences in the root-associated fungal communities of specific *Salix* clones as they may be indicators of their adaptability potential and disease susceptibility. Furthermore, this information could also be useful to evaluate the appropriateness of introducing foreign clones and their effects on native fungal populations.

3.3. Objectives and Hypothesis

The objectives of this study were to: *i*) assess the fungal diversity associated with the roots of willows grown under SRIC, *ii*) identify potentially pathogenic and beneficial fungi found among the root-associated fungal communities, and *iii*) investigate the influence of plant health status, plantation age, province and clone identity on root-associated fungal communities of promising *Salix* genotypes grown in the Canadian Prairies. These objectives were proposed to test the hypothesis that the dominance of pathogenic and beneficial fungi associated with the

roots of *Salix* spp. grown under SRIC is related to plant health status as well as with major characteristics of these agroforestry systems.

3.4. Materials and Methods

3.4.1. Study Sites

Two studies were performed to investigate the changes in the fungal communities living in the roots of willows grown in SRIC systems:

Study 1 investigated the fungal communities associated with the *Hotel* (*S. purpurea* L.) clone in plantations established in two different years and in two different provinces. Four sites were sampled. Sites 1 and 2 were both situated in Alberta (AB) with the former being planted in 2006 and the latter in 2005. Sites 3 and 4 were both in Saskatchewan (SK) and were planted in 2006 and 2005, respectively (Table 3-1). Study 2 compared fungal communities associated with the roots of seven different willow clones. All clones were grown in Site 5, which was established in SK in 2006 (Table 3-1). The clones included in this study were: *Hotel*, *Charlie* (probably *S. alba* x *glatfelteri*), *India* (*S. dasyclados* Wimm.), *Juliet* (*S. eriocephala* Michx.), *SV1* (*S. dasyclados* Wimmer), *SX61* (*S. sachalinensis* Fr. Schm.) and *SX64* (*S. miyabeana* Seemen).

All sites were situated in plantations included in the project for the development of willows for agroforestry and bioenergy in Canada. The characteristics of each site are presented in Table 3-1. Before the establishment of willows, all sites had been cultivated with cereals. The sites are classified as moderate and high quality lands. In all sites, willows were distributed according to a standard design: three row beds at 60 by 60 cm of tree spacing with 200 cm between beds, and an average of 150 plants per bed (15625 plants per ha) (Volk *et al.* 2004).

3.4.2. Experimental Design and Sampling

For both studies root samples were collected in the summer of 2007 from randomly chosen plants. In Study 1 the experimental design combined three variables with two levels each: health status (healthy and diseased), province (AB and SK) and year of establishment (2005 and 2006). Only plants of the *Hotel* clone were sampled. Study 2 focused on the differences related with health status and clone identity; therefore, the experimental design combined two levels of

Table 3-1. Site characteristics of *Salix* spp. plantations where root-associated fungal communities were studied.

	Province	Site name	Coordinates	Texture	pH	Carbon (%)	Nitrogen (%)	Year of establishment	Mean annual precipitation (mm) [§]	Clone
Study 1	Saskatchewan (SK)	Site 1	UTM 12U E0389931.8, N5776381.7	Heavy clay	6.5-7.5	2.0-2.6	0.18-0.30	2006	348.3	Hotel
		Site 2	UTM 13U E467155.03 N5872599.0	Clay overlying sandy loam	8.1-8.5	2.3-3.9	0.19-0.28	2005	348.3	
		Site 3	UTM 12U E0330943, N5921366	Clay	5.5-7.5	3.9-8.1	NA	2006	459.6	
		Site 4	UTM 12U E0330943, N5921366	Clay	5.5-7.5	3.9-8.1	NA	2005	459.6	
Study 2	Saskatchewan (SK)	Site 5	UTM 12U E0389931.8, N5776381.7	Heavy clay	6.5-7.5	2.0-2.6	0.18-0.30	2006	348.3	Charlie, Hotel, India, SV1, Juliet, SX64, SX61

[§] Climate average for the last 30 years. Environment Canada (2010).

NA: Not available.

health status (healthy and diseased) and seven different clones (*Charlie*, *Hotel*, *India*, *Juliet*, *SV1*, *SX61* and *SX64*).

Diseased plants were considered when they exhibited the following symptoms: limited growth, chlorosis, stem canker close to the root and root rot.

Three plants were sampled for each resultant combination of variables. An auger (capacity $5.03 \times 10^3 \text{ cm}^3$) was used to take a sample 30 cm apart from the stem, taking the first 20 cm of soil to ensure that the sample belonged to the selected plant. The samples were taken from the area in between the rows of trees. A portion of secondary and fine roots was recovered and gently shaken to clean excessive soil before being placed in plastic bags. The samples were stored at 4°C before the isolation of fungi.

3.4.3. Data Collection

In order to preferentially isolate endophytes, each sample was surface sterilized after a rinse with tap water. The sterilization protocol started with immersion in sterile distilled water (SDW) for one minute followed by ethanol (95%) for ten seconds, SDW for ten seconds, bleach (5%) for one minute, SDW for one minute and a final rinse with SDW for one minute. The sterilized roots were air dried in a laminar flow chamber and cut into pieces 1 to 1.5 cm long. Five pieces were placed on growth media supplemented with antibiotics (streptomycin sulphate 100 mg L^{-1} and neomycin sulphate 12 mg L^{-1}) (Yergeau *et al.* 2006). Three repetitions were performed for each sample.

The growth media used for the fungal isolation were: potato dextrose agar (BD and Co.), synthetic potato dextrose agar (BD and Co.) and Modified Melin Norkrans. The Petri dishes were incubated at room temperature (20°C) and darkness. The dishes were monitored for four to eight weeks to isolate each fungus as soon as it started emerging from the root fragment. Pure cultures were established using standard microbiological procedures.

3.4.4. Identification of Fungal Taxa

Pure cultures were grouped into operational taxonomic units (OTU) based on their morphology and with the aid of microscopy for doubtful cultures (Vujanovic *et al.* 2007). All isolates have been deposited in the Saskatchewan Microbial Collection and Database under

accession numbers SMCD2301-2400. For each OTU, DNA extraction and sequencing were performed as described below.

For DNA extraction, the mycelium collected with a sterile scalpel was ground in liquid nitrogen before using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer instructions. For taxonomical identification, PCR was performed in a final volume of 25 μ L, using as template the extracted DNA and the conditions below. The internal transcribed spacers (ITS) rDNA region was targeted using the fungal-specific primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990) (initial denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 57°C for 35 s and 72°C for 45 s and a final extension at 72°C for 10 min). In cases for which the amplification of the ITS region was not possible, different pairs of primers were used, for instance: NS1 and NS6 (White *et al.* 1990), which targets the nuclear small-subunit rDNA sequence, or EF1 and EF2 (Yergeau *et al.* 2005), which amplifies the α -elongation factor gen. The conditions used were the same as published by the respective authors.

The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and submitted for sequencing at the Plant Biotechnology Institute (Saskatoon, Saskatchewan). Subsequently, similarity analyses were performed using the Basic Local Alignment Search Tool (BLAST) available from NCBI (<http://www.ncbi.nlm.nih.gov>). Sequences were deposited in the GenBank database under the accession numbers: GU934498-GU934571 (sequences amplified with primers ITS1-F/ITS4), GU934572-GU934575 (sequences amplified with primers NS1/NS6) and GU934576-GU934577 (sequences amplified with primers EF1/EF2).

3.4.5. Statistical Analyses

Both the Shannon's diversity index ($H' = - \sum p_i \times \log_{10}(p_i)$) and the equitability ($E = H'/H_{\max}$) were calculated. The value of the Shannon's index calculated for empirical data is often between 1.5 and 3.5 (Magurran 2004). In this study, the value of H_{\max} was calculated as $\log_{10}(S)$, where S was the maximum number of taxa that were found in each resultant combination of health status, year, province and clone. Each taxon was taxonomically classified according to the Index Fungorum database (www.indexfungorum.org).

Matrices of similarity were calculated with the Jaccard's coefficient, using the presence/absence data. These matrices were later submitted to SYSTAT 12 (SYSTAT Software, Inc.) to generate cluster trees using the unweighted pair-group arithmetic average (UPGMA) method.

Abundance data were root-square transformed and submitted to CANOCO (Windows version 4.5) (ter Braak and Smilauer 2002) for ordination analyses. Correspondence and Canonical Correspondence Analyses were performed for each study. The analyses were focused on inter-taxa distances and rare taxa were underweighted. The tests of significance were performed with 999 permutations.

Published ITS sequences representing major fungal orders (James *et al.* 2006; Visentin *et al.* 2009), together with ITS sequences obtained in this work were aligned with Clustal W included in MEGA 4.1 software (Tamura 2007). A phylogenetic analysis was performed using the Maximum Likelihood approach with TOPALi (version 2.5) software (Milne 2004). The substitution model used was HKY (Hasegawa *et al.* 1985). Tree reliability was estimated by a bootstrap test with 100 repetitions.

3.5. Results

3.5.1. Fungal Diversity in the Roots of Willows Grown under SRIC

The isolation and quantification of cultivable fungi from the roots of willows planted in SK and AB showed that fungal communities from diseased plants tended to be more diverse than those from healthy plants (Table 3-2). The majority of communities isolated from diseased plants had higher values of diversity, as calculated by the Shannon's index (H'), than their healthy counterparts. The most outstanding difference was observed in Study 2 for the *Juliet* clone, where the diversity calculated for the fungal community of diseased plants was 1.32 whereas for healthy plants of the same clone it was only 0.75 (Table 3-3).

The fungal communities were characterized by an even distribution of taxa, as demonstrated by the equitability values. The majority of taxa in each community were characterized by a frequency of isolation less than 6% and only a few were found to be highly frequent throughout all the treatments. In both studies, the values of equitability were higher than 0.5 (Table 3-2 and Table 3-3). The values of equitability corresponded with the values of

Table 3-2. Abundance, diversity and equitability of fungal communities inhabiting the roots of the *Hotel* clone according to plant health status, province and year of establishment.

	Diseased				Healthy			
	Alberta		Saskatchewan		Alberta		Saskatchewan	
	2005	2006	2005	2006	2005	2006	2005	2006
Year of establishment								
Total number of cultures isolated	36	21	26	49	11	18	32	35
Number of taxa	18	11	14	14	8	12	10	13
Shannon diversity (H')	1.13	0.97	1.06	0.93	0.86	1.04	0.79	0.98
Evenness (E)	0.85	0.76	0.8	0.66	0.65	0.81	0.59	0.71

Table 3-3. Abundance, diversity and equitability of fungal communities inhabiting the roots of seven *Salix* clones grown under short rotation intensive culture in a plantation established in 2006 in Saskatchewan.

Clone	Diseased							Healthy						
	Charlie	Hotel	India	Juliet	SV1	SX61	SX64	Charlie	Hotel	India	Juliet	SV1	SX61	SX64
Total number of cultures isolated	36	49	33	36	12	32	37	23	35	22	9	16	31	51
Number of taxa	24	14	15	24	9	20	14	12	13	12	6	9	14	24
Shannon diversity (H')	1.3	0.93	1.09	1.32	0.91	1.21	1.05	0.97	0.98	1.03	0.75	0.9	1.06	1.28
Evenness (E)	0.89	0.66	0.81	0.91	0.84	0.86	0.73	0.66	0.71	0.77	0.52	0.83	0.75	0.9

diversity, i.e. communities with low diversity had also low equitability, and communities with high diversity had high equitability.

In Study 1, the fungal community from roots of diseased plants from AB 2005 was the most diverse ($H' = 1.13$) whereas the community from healthy plants from SK 2005 had the lowest value ($H' = 0.79$) (Table 3-2). In Study 2, the highest fungal diversity was found in diseased plants of the *Juliet* clone followed by diseased plants of *Charlie* whereas the lowest was calculated for healthy plants of the *Juliet* and *SVI* clones (Table 3-3).

3.5.2. Taxonomical Groups of Fungi Associated with Willow Roots

The isolates obtained in both studies were further classified into 88 taxa, representing 42 fungal genera. Most of the isolates belonged to the phylum Ascomycota, some Zygomycota and only few Basidiomycota (Table 3–4). Two families, Trichocomaceae and Nectriaceae, had the highest species richness, being represented by a higher number of taxa than the rest of the families (Table 3–4).

All taxa were taxonomically identified using BLAST, selecting matches with similarity higher than 98% to sequences in the GenBank database (E -value <0.05) (Table 3–4). However, 14 taxa presented low matching similarity ($<98\%$) and their morphological characteristics were not suitable to allow definitive identification. Therefore, their taxonomic identity remained uncertain. The taxon with the lowest percentage of similarity was identified as an uncultured soil fungus of which the closest match was *Inocybe curvipes* P. Karst. (82%). Other isolates that were identified with a similarity less than 98% were *Eucasphaeria capensis* Crous (91%), *Dothidotthia aspera* (Ellis & Everh.) M.E. Barr (92%), *Byssochlamys nivea* Westling (94%), *Chaetomium* sp. strain 4 (94%), *Chaetomium* sp. strain 3 (96%), *Fusarium* sp. strain 3 (96%), *Mortierella gamsii* Milko (96%), *Mortierella indohii* C.Y. Chien (96%), *Umbelopsis ramanniana* W. Gams (96%), *Chaetomium* sp. strain 1 (97%), *Cytospora chrysosperma* (Pers.) Fr. strain 2 (97%), *Leptosphaeria* sp. strain 2 (97%) and *Nectria* sp. (97%).

The amplification of the ITS region produced low quality sequences in six taxa, hence other regions were targeted (Table 3-4). *Didymella cucurbitacearum* A.J. Roy, *Dothidotthia aspera*, *Fusarium oxysporum* E.F. Sm. & Swingle strain 1 and *Mortierella indohii* were all identified targeting the small subunit rDNA region with the primer pair NS1/NS6. Their

similarities to the GenBank sequences were 98, 92, 98 and 96%, respectively. Similarly *F. redolens* Wollenw. strains 1 and 2 were identified targeting a partial region of the alpha elongation factor gene with the primer pair EF1/EF2. The similarities to the GenBank sequences were 99 and 98%, respectively.

An unrooted phylogenetic tree was generated with sequences obtained from the amplification of the ITS rDNA region (Figure 3-1). The family with the highest number of taxa was Nectriaceae (28 taxa) followed by Trichocomaceae and Pleosporaceae (11 and 10 taxa, respectively). Following were Hypocreaceae, Chaetomiaceae and the Incertae sedis group of Leotiomyces (six taxa each); Valsaceae and Leptosphaeriaceae (four taxa each); Diaporthaceae, and the Incertae sedis groups of Helotiales and Trichosphaeriales (three taxa each); Bionectriaceae, Mucoraceae and Mortierellaceae (two taxa each). The remaining nine families were represented only by one taxon.

Two isolates could not be morphologically identified and the best matches in the GenBank database were to uncultured endophytic fungi. In the phylogenetic analysis, one of them (OTU# 970) was positioned among the group of Pleosporales and the other (OTU# 1040) was placed close to Entomophthorales and other orders of Zygomycota (Figure 3-1).

3.5.3. Composition of Fungal Communities in Relation to Year of Establishment, Province and Plant Health Status

In order to reveal the potential patterns underlying the changes in the composition of fungal communities, a correspondence analysis (CA) was performed on the data of each study. The biplot, produced by means of such analysis with the data from Study 1 (Figure 3-2), indicated that two variables, i.e. health status and year of establishment, could be related with the variation associated with the first axis. Province, the third variable of interest for this study, appeared to be related with the second axis. The amount of variation explained by the first and second axes was 20.7% and 18.8%, respectively. From the CA it was also noted that the diseased status was mostly associated with young plants (planted in 2006) rather than with the older ones (planted in 2005).

Despite the overall high fungal diversity that was recovered, certain differences were found regarding the isolation frequency or dominance of particular taxa in each sample.

Table 3-4. Taxonomical identification of fungi isolated from roots of *Salix* spp. grown under short rotation intensive culture. Sequence analyses were performed using the basic local alignment search tool (BLAST). The targeted DNA regions and the percent identity to published sequences are presented.

Description	Total number of cultures isolated	OTU*	DNA sequence [‡]	Assigned accession number	Most closely related sequence in the GenBank database	Percent identity
Phylum Ascomycota						
Diaporthales, Diaporthaceae						
<i>Phomopsis columnaris</i> D.F. Farr & Castl. strain 1	12	370	ITS	GU934561	DQ093770.1	99%
<i>Phomopsis columnaris</i> D.F. Farr & Castl. strain 2	36	410	ITS	GU934562	AF439625.1	99%
Diaporthales, Valsaceae						
<i>Cytospora chrysosperma</i> (Pers.) Fr. strain 1	1	500	ITS	GU934515	EU918709.1	99%
<i>Cytospora chrysosperma</i> (Pers.) Fr. strain 2	5	700	ITS	GU934516	EU918709.1	97%
Eurotiales, Trichocomaceae						
<i>Aspergillus wentii</i> Wehmer	1	760	ITS	GU934502	AY373884.1	99%
<i>Byssosclamyces nivea</i> Westling	1	960	ITS	GU934506	DQ322220.1	94%
<i>Penicillium citreonigrum</i> Dierckx strain 1	2	600	ITS	GU934550	EU497959.1	98%
<i>Penicillium citreonigrum</i> Dierckx strain 2	1	630	ITS	GU934551	EU497959.1	99%
<i>Penicillium janthinellum</i> Biourge strain 1	5	610	ITS	GU934553	AY373921.1	99%
<i>Penicillium janthinellum</i> Biourge strain 2	1	1050	ITS	GU934554	AJ608945.1	99%
<i>Penicillium vinaceum</i> J.C. Gilman & E.V. Abbott	4	620	ITS	GU934557	EU833227.1	99%
<i>Penicillium coprobium</i> Frisvad	6	640	ITS	GU934552	DQ339559.1	99%
<i>Penicillium thomii</i> Maire	2	740	ITS	GU934556	AY373934.1	99%
<i>Penicillium</i> sp.	9	870	ITS	GU934555	EU128592.1	100%
Helotiales, Sclerotiniaceae						
<i>Botryotinia fuckeliana</i> (de Bary) Whetzel	1	850	ITS	GU934505	EF207415.1	99%

Helotiales, Vibrisseaceae						
<i>Phialocephala fortinii</i> C.J.K. Wang & H.E. Wilcox	5	110	ITS	GU934559	AY394921.1	99%
Hypocreales, Hypocreaceae						
<i>Trichoderma viride</i> Pers.	3	220	ITS	GU934567	DQ846665.1	99%
<i>Hypocrea viridescens</i> Jaklitsch & Samuels	5	230	ITS	GU934535	EU280104.1	99%
<i>Hypocrea lixii</i> Pat. strain 1	12	660	ITS	GU934532	AY605732.1	99%
<i>Hypocrea lixii</i> Pat. strain 2	1	680	ITS	GU934533	EU280077.1	99%
<i>Hypocrea lixii</i> Pat. strain 3	5	690	ITS	GU934534	EU280092.1	99%
Hypocreales, Incertae sedis						
<i>Gliomastix</i> sp. §	2	120				
<i>Acremonium crotocinigenum</i> (Schol-Schwarz) W. Gams	6	270	ITS	GU934498	AJ621773.1	99%
Hypocreales, Nectriaceae						
<i>Fusarium culmorum</i> (W.G. Sm.) Sacc. strain 1	4	160				
<i>Fusarium culmorum</i> (W.G. Sm.) Sacc. strain 2	33	180	ITS	GU934521	AY147321.1	99%
<i>Fusarium</i> sp. strain 1 §	1	170				
<i>Fusarium</i> sp. strain 2 §	2	190				
<i>Fusarium</i> sp. strain 3	1	440	ITS	GU934526	AY188917.1	96%
<i>Fusarium</i> sp. strain 4	1	930	ITS	GU934527	DQ459866.1	98%
<i>Fusarium</i> sp. strain 5	1	1100	ITS	GU934528	EU750678.1	99%
<i>Fusarium</i> sp. strain 6	1	1120	ITS	GU934529	EU750695.1	98%
<i>Fusarium redolens</i> Wollenw. strain 1	28	240	EF	GU934576	DQ854915.1	99%
<i>Fusarium redolens</i> Wollenw. strain 2	61	250	EF	GU934577	DQ854915.1	98%
<i>Fusarium redolens</i> Wollenw. strain 3	11	280	ITS	GU934525	X94169.1	99%
<i>Fusarium oxysporum</i> E.F. Sm. & Swingle strain 1	26	260	NS	GU934574	AB110910.1	98%
<i>Fusarium oxysporum</i> E.F. Sm. & Swingle strain 2	3	330	ITS	GU934524	EU839400.1	99%
<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> W.C. Snyder & H.N. Hansen	1	1080	ITS	GU934523	DQ535184.1	98%
<i>Fusarium solani</i> (Mart.) Sacc.	4	290				
<i>Fusarium equiseti</i> (Corda) Sacc. strain 1	2	340	ITS	GU934522	AY147362.1	99%

<i>Fusarium equiseti</i> (Corda) Sacc. strain 2 §	46	350				
<i>Gibberella avenacea</i> R.J. Cook strain 1	26	140	ITS	GU934530	EU255801.1	99%
<i>Gibberella avenacea</i> R.J. Cook strain 2	7	150	ITS	GU934531	EU255801.1	100%
<i>Nectria</i> sp.	2	590	ITS	GU934544	AY805575.1	97%
<i>Neonectria radiculicola</i> (Gerlach & L. Nilsson) Mantiri & Samuels strain 1	16	200	ITS	GU934545	AJ875336.1	99%
<i>Neonectria radiculicola</i> (Gerlach & L. Nilsson) Mantiri & Samuels strain 2	1	1000	ITS	GU934546	AJ875334.1	99%
<i>Neonectria radiculicola</i> (Gerlach & L. Nilsson) Mantiri & Samuels strain 3	4	360	ITS	GU934547	AJ875331.1	99%
<i>Neonectria radiculicola</i> (Gerlach & L. Nilsson) Mantiri & Samuels strain 4	3	540	ITS	GU934548	AJ875331.1	98%
Hypocreales, Bionectriaceae						
<i>Bionectria ochroleuca</i> (Schwein.) Schroers & Samuels	2	430	ITS	GU934503	AF106532.1	99%
Incertae sedis, Glomerellaceae						
<i>Colletotrichum pisi</i> Pat.	2	560	ITS	GU934514	EU400150.1	99%
Leotiomycetes, Incertae sedis						
<i>Chaetomella</i> sp. §	2	130				
Pleosporales, Dothidotthiaceae						
<i>Dothidotthia aspera</i> (Ellis & Everh.) M.E. Barr	2	210	NS	GU934573	EU673228.1	92%
Pleosporales, Incertae sedis						
<i>Didymella bryoniae</i> (Fuckel) Rehm	6	450	ITS	GU934518	AB266846.1	98%
<i>Didymella cucurbitacearum</i> A.J. Roy	2	570	NS	GU934572	AY293779.1	98%
<i>Periconia macrospinosa</i> Lefebvre & Aar.G. Johnson	2	310	ITS	GU934558	AJ246158.1	99%
<i>Pyrenochaeta</i> sp.	2	720	ITS	GU934564	AM921726.1	99%
<i>Phoma multirostrata</i> (P.N. Mathur, S.K. Menon & Thirum.) Dorenb. & Boerema	5	880	ITS	GU934560	EF585392.1	99%
Pleosporales, Leptosphaeriaceae						
<i>Leptosphaeria</i> sp. strain 1	5	860	ITS	GU934536	DQ093683.1	99%
<i>Leptosphaeria</i> sp. strain 2	6	530	ITS	GU934537	AJ317958.1	97%
Pleosporales, Pleosporaceae						
<i>Alternaria</i> sp. strain 1	26	100	ITS	GU934499	EF432293.1	99%

<i>Alternaria</i> sp. strain 2	2	890	ITS	GU934500	DQ779787.1	98%
<i>Alternaria</i> sp. strain 3	1	1090	ITS	GU934501	DQ779787.1	99%
<i>Cochliobolus sativus</i> (S. Ito & Kurib.) Drechsler ex Dastur	2	710	ITS	GU934513	EF452447.1	98%
<i>Bipolaris sorokiniana</i> (Sacc.) Shoemaker	2	730	ITS	GU934504	EU030351.1	100%
<i>Epicoccum nigrum</i> Link	1	1070	ITS	GU934519	AJ279448.1	98%
<i>Lewia infectoria</i> (Fuckel) M.E. Barr & E.G. Simmons	2	1030	ITS	GU934538	AY154718.1	99%
<i>Stemphylium globuliferum</i> (Vestergr.) E.G. Simmons	1	910	ITS	GU934566	AY372675.1	99%
<i>Dendryphion nanum</i> (Nees) S. Hughes	2	1020	ITS	GU934517	AY387657.1	99%
Pleosporales, Sporormiaceae						
<i>Preussia funiculata</i> (Preuss) Fuckel	1	1130	ITS	GU934563	AY943059.1	99%
Sordariales, Chaetomiaceae						
<i>Chaetomium</i> sp. strain 1	1	380	ITS	GU934508	EU750691.1	97%
<i>Chaetomium</i> sp. strain 2	5	420	ITS	GU934509	EF672314.1	98%
<i>Chaetomium</i> sp. strain 3	1	460	ITS	GU934510	EU750691.1	96%
<i>Chaetomium</i> sp. strain 4	1	940	ITS	GU934511	EU035795.1	94%
<i>Chaetomium</i> sp. strain 5	1	950	ITS	GU934512	AJ279468.1	99%
<i>Chaetomium globosum</i> Kunze	11	470	ITS	GU934507	AY429056.1	99%
Sordariomycetes, Incertae sedis						
<i>Eucasphaeria capensis</i> Crous (possibly <i>Myrothecium</i> sp.)	3	550	ITS	GU934520	EU272516.1	91%
Trichosphaeriales, Incertae sedis						
<i>Nigrospora oryzae</i> (Berk. & Broome) Petch	1	390	ITS	GU934549	EU272488.1	99%
Xylariales, Amphisphaeriaceae						
<i>Truncatella angustata</i> (Pers.) S. Hughes	6	320	ITS	GU934568	AF377300.1	99%
Xylariales, Incertae sedis						
<i>Microdochium</i> sp.	5	750	ITS	GU934541	AJ279477.1	99%
<i>Microdochium bolleyi</i> (R. Sprague) de Hoog & Herm.-Nijh. strain 1	1	580	ITS	GU934539	AJ279454.1	99%
<i>Microdochium bolleyi</i> (R. Sprague) de Hoog & Herm.-Nijh. strain 2	4	920	ITS	GU934540	AJ279475.1	99%

Phylum Basidiomycota						
Cantharellales, Ceratobasidiaceae						
<i>Rhizoctonia solani</i> J.G. Kühn	2	830	ITS	GU934565	EU591803.1	99%
Phylum Zygomycota						
Mucorales, Mucoraceae						
<i>Rhizopus</i> sp. [§]	23	770				
<i>Mucor circinelloides</i> Tiegh.	2	790	ITS	GU934543	DQ118987.1	99%
Mucorales, Umbellopsidaceae						
<i>Umbelopsis ramanniana</i> (Möller) W. Gams	2	980	ITS	GU934569	AB193533.1	96%
Mortierellales, Mortierellaceae						
<i>Mortierella indohii</i> C.Y. Chien	2	480	NS	GU934575	EU688965.1	96%
<i>Mortierella gamsii</i> Milko	4	490	ITS	GU934542	DQ093723.1	96%
Non identified						
Uncultured endophytic fungus	3	970	ITS	GU934570	EF505610.1	99%
Uncultured soil fungus	1	1040	ITS	GU934571	EU807355.1	82%

* OTU: Operational Taxonomic Unit.

‡ When the amplification of the ITS region was not successful other DNA regions were used for identification. ITS: internal transcribed spacers; EF: α -elongation factor (primer pair EF1/EF4); NS: nuclear small-subunit rDNA (primer pair NS1/NS6).

§ Identification based exclusively on morphology. No molecular analyses were performed.

In all cases *Fusarium* was the most abundant genus; however, its frequency of isolation decreased from about 38% in plants from 2006 to about 22% in plants from 2005. Similarly, the genus *Phomopsis* suffered a reduction from 2006 to 2005, going from a frequency of isolation in young plants of about 12% to an almost inexistent presence in older plants (0.7%). Among the isolated species belonging to *Fusarium* sp., the most dominant were *F. culmorum* (W.G. Sm.) Sacc. and *F. redolens*. Regarding *Phomopsis* sp. both of the isolated strains (*P. columnaris* D.F. Farr & Castl. strains 1 and 2) were equally dominant. A similar pattern was observed for isolates of *Microdochium bolleyi* (R. Sprague) de Hoog & Herm.-Nijh. and *Neonectria radiculicola* (Gerlach & L. Nilsson) Mantiri & Samuels which were found primarily associated with diseased plants from 2006 rather than with healthy and older plants.

In contrast to *Fusarium* sp., *Phomopsis* sp., *Microdochium* sp. and *Neonectria* sp., some genera increased in abundance in older plants. Thus, *Alternaria* sp. that accounted for only about 1% in 2006, increased in frequency to almost 14% in plants from 2005. Other genera with increased dominance in older plants include *Penicillium* and *Leptosphaeria*. Furthermore, in agreement with the suggested relationship between health status and year of plant establishment, these same genera were also more abundant in the healthy plants than in the diseased ones. From the different isolates belonging to these genera, *Alternaria* sp. strain 1, *Penicillium* sp. and *Penicillium coprobium* Frisvad were the most closely related to healthy plants.

New taxa appeared in the older plants but their relation with plant health status could not be clearly stated as their frequency of isolation was very low (generally less than 1%). Such taxa included isolates of *Epicoccum* sp., *Gliomastix* sp., *Mortierella* sp., and *Pyrenochaeta* sp. among others.

With respect to the variables health status and province, the isolates of *Neonectria* sp. (*N. radiculicola* strains 1, 2, 3 and 4) were particularly dominant among the fungal communities of diseased plants. Thus, *Neonectria* sp. accounted for about 15% of the taxa found in diseased plants, but it was only about 6% in healthy plants. In addition, the same genus was more abundant in plants from AB than in plants from SK.

Both *Rhizopus* sp. and *Chaetomium* sp. did not seem to be associated with plant health status or year of establishment since their frequency remained constant throughout these variables. However, *Rhizopus* sp. was isolated almost exclusively from AB, which suggests a high relationship with the variable province. On the other hand, differences in the isolates of

Chaetomium sp. were observed among provinces; thus, *C. globosum* was exclusively found in AB whereas *Chaetomium* sp. strains 2 and 3 were isolated only from SK.

Although the differences in the fungal communities between provinces were less evident than between year of establishment and health status, some taxa were observed to be present only in one of the two provinces. Thus, the isolates of *Hypocrea* sp., *Dydimella* sp., *Rhizoctonia* sp., and *Botryotinia* sp. were exclusively isolated from AB. On the contrary, isolates belonging to *Cytospora* sp., *Dendryphion* sp., *Eucasphaeria* sp., *Mortierella* sp. as well as other much less abundant (less than 1%), were exclusively found in SK.

3.5.4. Fungal Communities in Different Willow Clones

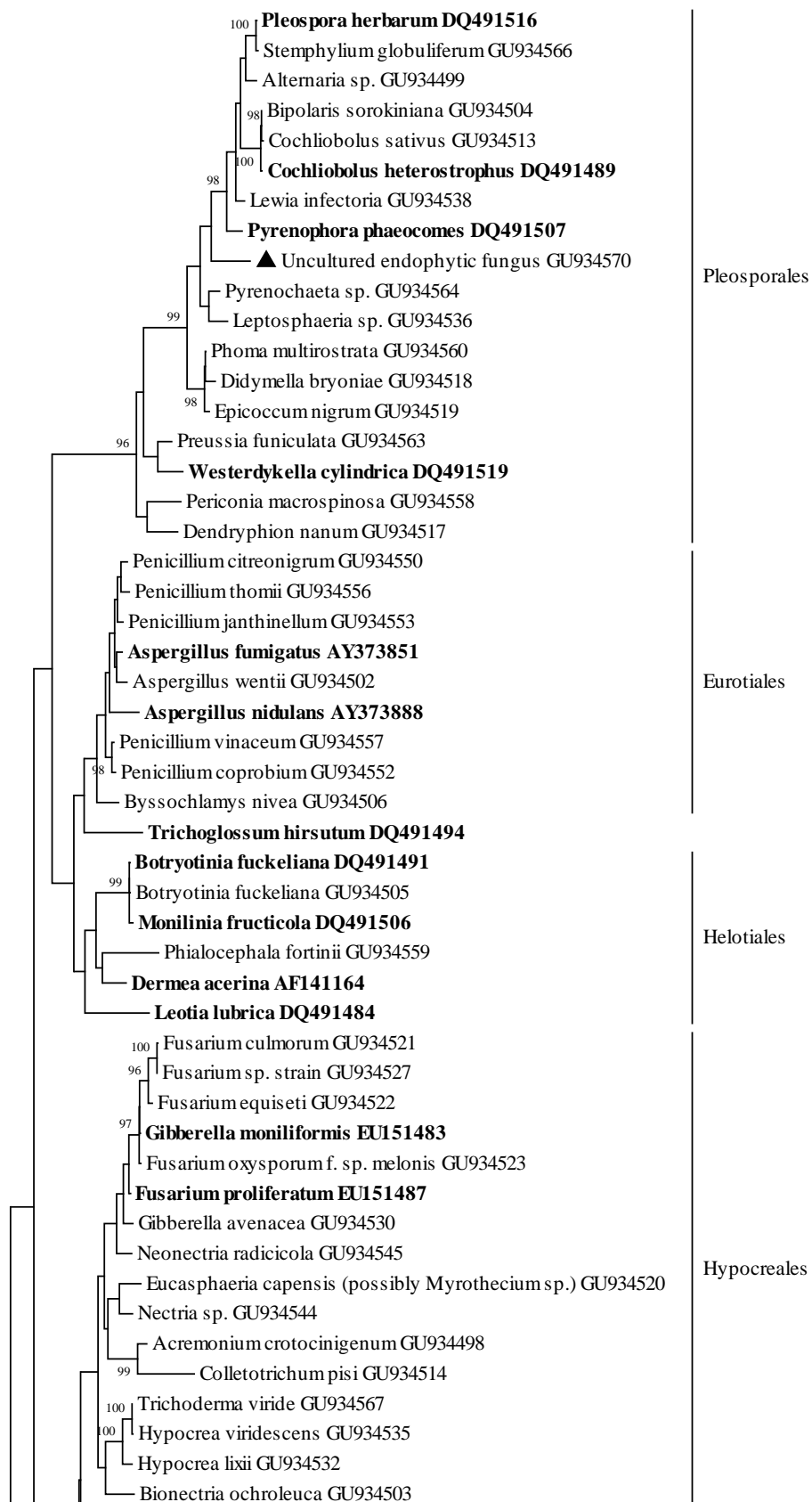
According to the CA of data from Study 2 (Figure 3-2), most of the variation observed in the fungal communities seemed to be associated with differences among clones rather than to differences between plant health status. The variation associated with the first axis was 15.6% and with the second was 12.5%.

Similar to the data obtained in Study 1, *Fusarium* sp. isolates were dominant in all cases. Other abundant isolates in the samples included those belonging to *Phomopsis* sp., *Gibberella* sp., and *Chaetomium* sp.

The CA biplot (Figure 3-2) presents a cluster of the *SX61*, *SX64*, *Charlie* and *Hotel* clones. The fungal communities of these four clones were dominated by isolates of *Fusarium* sp., *Gibberella* sp. and *Phomopsis* sp. with the latter being preferentially isolated from the *Hotel*, *SX61* and *SX64* clones. Furthermore, the similarities of these clones are also illustrated by the UPGMA tree (Figure 3-3), which supports more clearly the similarities of the communities of the four clones independently of the health status of the plants.

The clones *SX64* and *Charlie* seemed especially susceptible to be colonized by *Hypocrea* sp. (*H. lixii* Pat. strain 1), *Penicillium* sp. (*P. janthinellum* Biourge, *P. vinaceum* J.C. Gilman & E.V. Abbott, *P. coprobium*, and *P. thomii* Maire) and *Alternaria* sp. (*Alternaria* sp. strain 1), whereas the *SX61* clone appeared with a higher dominance of *Neonectria* sp. (*N. radicicola* strain 1), *Microdochium* sp. and *Chaetomium* sp. (*Chaetomium* sp. strains 4 and 5).

The communities associated with the *Hotel* and *India* clones shared a similar composition in relation with diseased plants. In both clones *Fusarium* sp. was less abundant in healthy plants than in the diseased ones. Thus, in diseased plants of *Hotel*, the isolates of *Fusarium* sp. (mainly



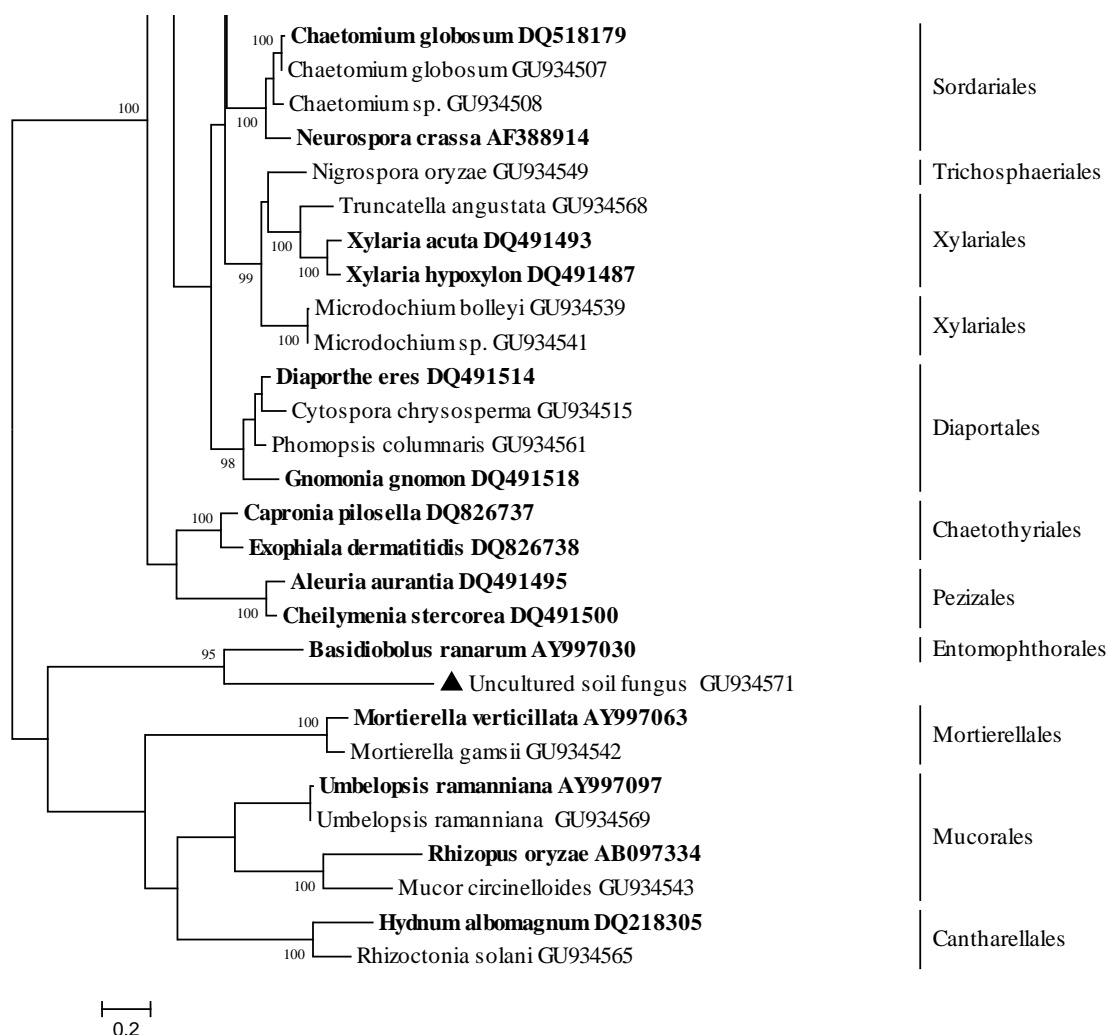


Figure 3-1. Unrooted phylogenetic tree using published ITS sequences (bold case) as well as sequences of fungi isolated in this work. The evolutionary history was inferred using the Maximum Likelihood method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The code next to the name refers to the accession number in the Gen Bank database (Table 3–4). Fungi isolated in this work that could not be taxonomically identified after BLAST search are indicated by a solid triangle (▲). Numbers in the branches are bootstrap values from 100 iterations; only values higher than 95 are presented.

F. culmorum, *F. redolens* and *F. oxysporum*) accounted for almost 80% of the total; by contrast, in healthy plants the same genus accounted for only 37% (principally *F. redolens*). Similarly, in the *India* clone, *Fusarium* sp. (*F. redolens*, *F. oxysporum* and *F. equiseti* (Corda) Sacc.) accounted for about 42% in diseased plants and only 27% in the healthy ones (*F. redolens* and *F. equiseti*). Diseased plants of both clones were also associated with *Truncatella* sp. while healthy plants were highly dominated by *Phomopsis* sp.

The fungal communities of the clones *Juliet*, *SVI* and *India* were characterized for substantial changes in their composition in relation to the health status of the plants. In addition, both *SVI* and *Juliet* clones were particularly different from the other clones (Figure 3-2) given the very low fungal diversity that they exhibited especially in healthy plants. Such communities were dominated by isolates belonging to *Fusarium* sp. (*F. redolens* strains 1 and 2, and *F. equiseti* strain 2), *Rhizopus* sp., *Chaetomium* sp. (*C. globosum*), and *Gibberella* sp. (*G. avenacea* R.J. Cook strains 1 and 2). In contrast, the diseased plants were found associated with *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox and *Mortierella indohii*. In the case of the diseased plants of the *Juliet* clone, they were also associated with *Alternaria* sp. (strains 2 and 3) and *Phomopsis columnaris* as well as with other less dominant taxa (frequency < 2.5%).

Canonical analyses were performed for both studies to test the statistical significance of the variables of interest. The results from these analyses were not significant ($P > 0.05$, Monte Carlo test) in spite of the high correlation observed between the extracted axes and the variables in the non-canonical analyses (correlation > 0.7).

3.6. Discussion

The study of root-associated fungal communities of willows grown in SRIC systems could enlighten key biological aspects influencing the successful development of these biomass crops in Canada. Most of the available studies with fungal communities in biomass plantations have been performed in sites with a minimum of five or six years of establishment (Baum and Hryniewicz 2006; Hutchison 1999; Khasa *et al.* 2002; Puttsepp *et al.* 2004; Vujanovic and Labrecque 2002). This work, however, focused on the years immediately after planting the cuttings, during which management practices are critical for plant development.

The results obtained in this work indicated that plants that were in their first year of establishment (planted in 2006) were more susceptible to be associated with disease-related fungi

a

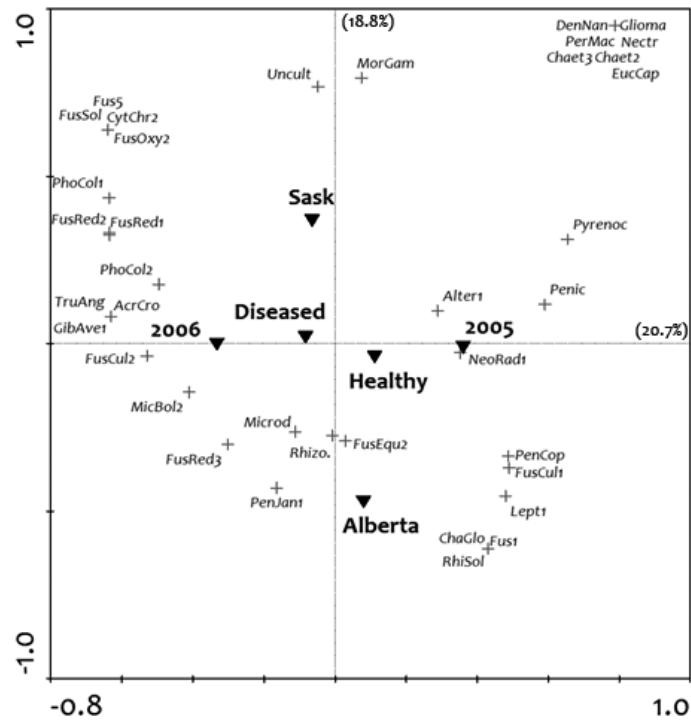
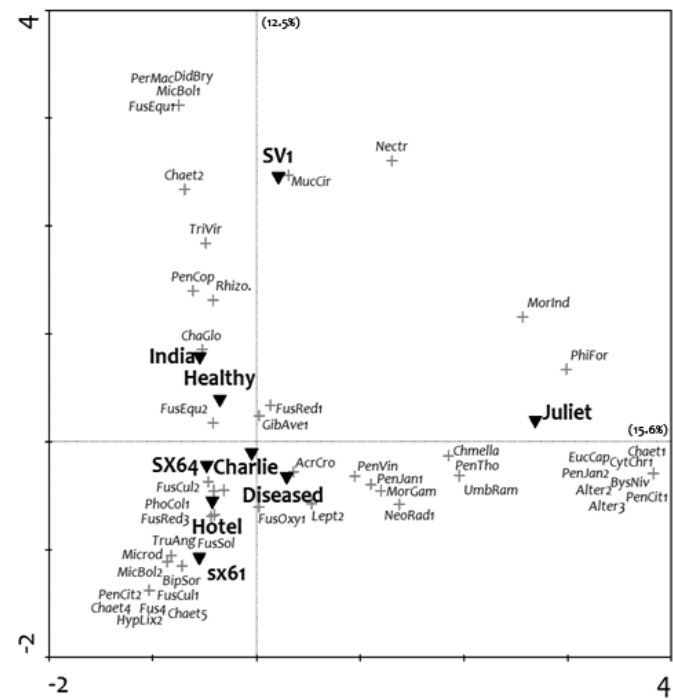
**b**

Figure 3-2. Correspondence Analysis (CA) of fungal communities associated with roots of *Salix* spp. grown under short rotation intensive culture **a)** CA of the relationship between plant health status (*Diseased* and *Healthy*), province (*Alberta* and *Sask*: Saskatchewan) and year of plantation establishment (2005 and 2006) and the associated fungal species. For clarity purposes only species with a fit between 20 and 100% are presented **b)** CA of the relationship between *Salix* clones (*India*, *SV1*, *Juliet*, *Charlie*, *SX64*, *SX61* and *Hotel*), health status and the associated fungal species. For clarity purposes only species with a fit between 10 and 100% are presented. The amount of variation associated with each axis is presented inside the brackets. The species names are abbreviated using the first three letters of the genus followed by the first three letters of the epithet and the strain number. The indices (coordinates) derived from a correspondence analysis inform simultaneously which species have important weight for each variable, and which variables have important weight for each species. Therefore, the proximity of any given species to a variable in the biplot may be interpreted as the variable having a particularly important weight in that species and vice versa. The origin of the coordinate system represents the average of variables and species, and the cumulated percentage of variance is used to evaluate the quality of the biplot and its suitability for interpretation (Hardle and Simar 2007; Leps and Smilauer 2003).

than plants that were in their second year (planted in 2005). This could be a direct consequence of using previously arable land, since during the initial stage of development the young root system is directly exposed to the fungal inoculum remaining in the soil from previously existent crops. Potentially deleterious fungi may constitute an important component of the remnant fungal soil community even after the previous crops have been removed. Traditional agricultural systems are known to be susceptible to a variety of diseases and management practices can have a negative effect on the diversity of natural populations of some beneficial fungi (Helgason *et al.* 1998). Hence, although willows are very well adapted to thrive in nutrient poor environments (Kuzovkina and Quigley 2005), their characteristics may not be suitable to withstand the initial pressure of settled pathogenic fungal populations. This situation however, may be overcome after the first year, once the roots penetrate the soil and the nutritional status of the young plants is improved. Furthermore, the permanent establishment of willows triggers a series of changes in the surroundings and will eventually modify the physical and chemical characteristics of the soil (Makeschin 1994) which in turn will directly influence the fungal diversity left after the removal of previous crops (Kahle *et al.* 2005).

The fungi isolated in this study included a high number of fast-growing ascomycetous species that are not commonly reported as related to roots of willows in natural environments. Similar fungal taxa have also been isolated in previous studies in biomass crops (Slapokas and Granhall 1991; Vujanovic and Labrecque 2002) which may be an indication of the necessity of promoting fungal populations that are naturally beneficial to willows when starting a SRIC. This is especially true since many of the isolated taxa are commonly referred to as phytopathogens in different crops and include species of *Fusarium* sp. (teleomorph: *Gibberella*), *Neonectria* sp., *Phomopsis* sp., *Didymella* sp. and *Cytospora* sp. (teleomorph: *Valsa*). Although some of these taxa are not reported as specific *Salix* pathogens, they may act opportunistically and use the newly planted cuttings as temporal hosts limiting their development. The risk of this type of interaction could also require further attention and even special practices during the site preparation and planting stages to decrease the chances of a "host-jump" event (Woolhouse 2005). Examples of new fungal diseases appearing in woody species have been observed in plants of *Eucalyptus* sp. and *Pinus* sp. following human introduction for forestry purposes (Coutinho 1998; Kinloch 2003). In this scenario, the hosts are highly susceptible given the absence of defense strategies against the pathogens resultant from the lack of a previous

exposure of both organisms. Therefore, the decrease of the pressure exerted by pre-existent fungal communities, in addition to adequate maintenance practices following planting, could greatly contribute to the successful establishment and development of the young willow cuttings.

In general, willow root diseases are not regarded as problematic for SRIC as opposed to diseases in leaves and stems (Hubbes 1983); however, the high abundance of *Fusarium* sp. and *Neonectria* sp. in roots of diseased plants, principally during the first year, could warrant further precautions. Different species of *Fusarium* have been previously isolated in studies with *Salix* spp. (Hubbes 1983; Slapokas and Granhall 1991), but no reports were found regarding the presence of *Neonectria* sp. Harmful species in the Fusaria-complex are usually strong producers of mycotoxins and different degrees of pathogenicity exist at the strain level (Desjardins 2006). In this work *Fusarium* species included isolates of *F. culmorum*, *F. equiseti*, *F. redolens* and *F. oxysporum* with the last one being most commonly isolated from diseased plants than from the healthy ones. Despite the general high dominance of this group, a reduction in its relative frequency was observed in older and healthy plants compared to the younger and diseased ones. This could be the result of the lack of specificity for *Salix* spp. as host and/or the interaction with other fungi that are better competitors for the resources available in the new evolving environment.

In fact, despite the high initial pressure of opportunistic fungi over the young cuttings, it seemed that over time plants were able to preferentially associate with potentially beneficial fungi. Such fungi could play a role in increasing the tolerance to the harsh conditions encountered during the initial stages of the SRIC establishment. Due to their increased dominance in healthy plants, *Alternaria* sp., *Penicillium* sp. and *Phomopsis* sp. appear to be some of the genera that may be studied for their potential benefits on willow cuttings.

The constant isolation of *Alternaria* sp. in healthy leaves of *Salix* spp. was also previously suggested by Vujanovic and Labrecque (2008) as an indication of its potential role as beneficial fungi. However, the authors also recognized the need for more supporting evidence as the majority of reports of *Alternaria* species are associated with damages in traditional crops (Akamatsu 1999). In a similar way, although a positive functional role cannot be dismissed for isolates of *Phomopsis* sp., more scientific evidence is still required. Many species belonging to this genus are known for causing plant diseases in agricultural systems (Rossman 2007); however, in woody plants *Phomopsis* sp. has been repeatedly found among the dominant genera

of endophytic fungi. Moreover, in some cases the association of woody plants with *Phomopsis* species has been suggested as an evolutionary adaptation for defense against plant predators (Rossman 2007).

Other types of benefits that may be obtained by willows when associated with these fungi could be directly related with the nutritional status of the plants. Species of *Penicillium*, including those isolated in this work (*P. janthinellum*), have been previously associated with the dynamics of phosphorus in the soil of *Salix* spp. (Baum and Hryniewicz 2006; Baum *et al.* 2006; Chatli *et al.* 2008) and some species have also been studied for their potential as biocontrol agents of *Fusarium* sp. (De Cal *et al.* 2000; Fang and Tsao 1995).

In addition to health status and year of establishment, site characteristics can also influence the composition of the willow root fungal communities. The communities from AB were highly dominated by potentially pathogenic fungi (*Fusarium* sp., *Neonectria* sp., *Rhizopus* sp., *Leptosphaeria* sp. and *Chaetomium* sp.) whereas the sites in SK exhibited both potential pathogenic and beneficial fungi, with *Fusarium* isolates accounting for the highest proportion of potentially pathogenic fungi. Differences among sites were previously found to influence the shape of the fungal communities associated with different crops and plant organs including leaves and bark of *S. viminalis* in eastern Canada (Vujanovic *et al.* 2006; Vujanovic and Labrecque 2008). Also, root-associated fungal communities in roots of *S. arctica* Pallas were affected by the physicochemical properties specific to each site (Fujimura *et al.* 2008). In the case of biomass plantations, differences among sites could also be related to the agricultural history of the sites. In Canada, crops preceding willow SRIC typically include wheat, barley, oats and canola. Fertilization and other management practices of these crops as well as of the newly established SRIC could result in changes in the composition of the fungal communities. For example, changes of fungal communities influenced by chemical fertilization have been reported in *Pinus* sp. forests fertilized with ammonium nitrate and urea (Arnebrant *et al.* 1990). The particular characteristics of each site differentially affect the fungal diversity and could have implications in the establishment of SRIC thus requiring management practices to be more site-specific (Makeschin 1994).

Different willows clones have been obtained through breeding and selection of parental individuals with a stock of desirable characteristics such as high biomass production, disease resistance, climatic adaptation and others (Begley *et al.* 2008; Kopp 2001; Labrecque and

Teodorescu 2005). Previous studies (Hunter *et al.* 1996; Labrecque and Teodorescu 2005) observed variability in the susceptibility of different willow clones to diseases. Hunter *et al.* (1996) observed that clones obtained from *S. caprea* L. or *S. aurita* L. were particularly susceptible to infection by *Melampsora caprearum* Thüm, while Baum and Hryniewicz (2006), reported differences in the degree of colonization by mycorrhizas in roots of two willow clones. These observations have been suggested to be related to a differential receptivity of the genotypes.

Unique characteristics of the structure of fungal communities associated with the roots of different clones were also observed in this work. *Hotel* (*S. purpurea*), *India* (*S. dasyclados*), *SVI* (*S. dasyclados*) and *Juliet* (*S. eriocephala*) clones were obtained from species that are originally from or have been already naturalized in North America (Kopp 2001). In particular, *SVI* has been largely studied in the United States and is used in SRIC plantations as a standard clone for biomass production. The fungal communities associated with each one of these four clones were specific to plant health status. Thus, for example, diseased plants of *Hotel* and *India* were highly dominated by *Fusarium* sp. whereas in healthy plants of the same clones the abundance of *Fusarium* sp. was comparatively reduced. At the same time potentially beneficial fungi that increased in abundance in these two clones were isolates of *Alternaria* sp., *Penicillium* sp. and *Phomopsis* sp.

The low fungal diversity found in the roots of the *Juliet* and *SVI* clones could be an indication of their resistance to fungal infection. This low susceptibility to fungal colonization has been previously reported for *S. dasyclados* (Baum and Hryniewicz 2006), the parental species of the *SVI* and *India* clones. This feature may, therefore, be related with genetic defense mechanisms present in *S. dasyclados* and transmitted onto its progeny (Ronnberg-Wastljung *et al.* 2008). The results of this work suggested, however, that in the *India* clone once those defense mechanisms are broken, plants are left highly susceptible to the invasion of numerous potentially pathogenic fungi.

In the case of the *Juliet* and *SVI* clones the potentially pathogenic fungi presenting an important threat appeared to be *Gibberella/Fusarium* sp. and *Phialocephala* sp. According to their abundance in diseased plants *Alternaria* sp. could also be pathogenic for *Juliet* whereas *Nectria* sp. could be for *SVI*. Interestingly, *Phialocephala* sp. isolates were recovered only from these two clones. Numerous and contradictory studies have been published regarding the role of

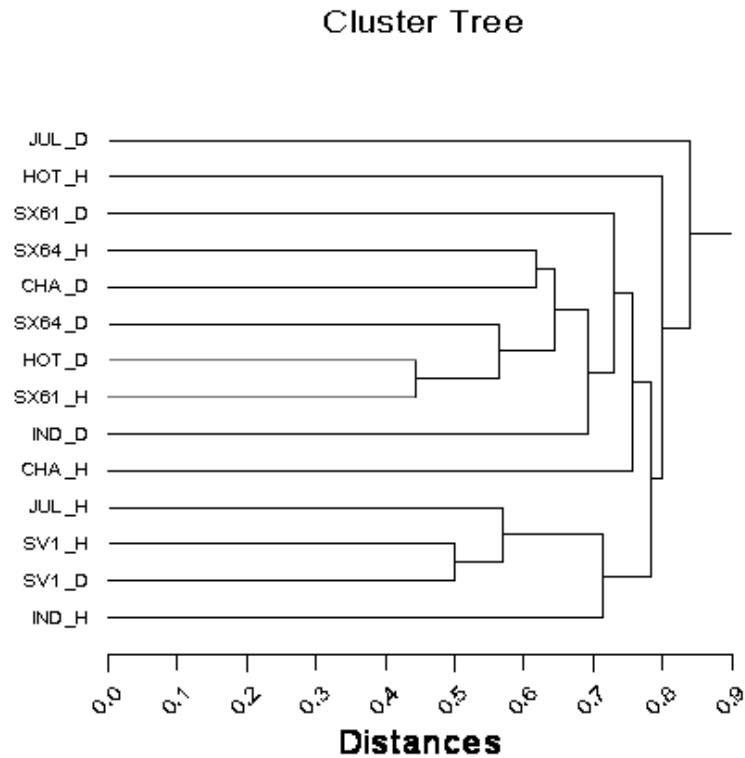


Figure 3-3. Unweighted arithmetic average clustering (UPGMA) of fungal communities isolated from healthy and diseased plants of seven *Salix* clones. Site located in Saskatchewan, planted in 2006. The distance used is the Jaccard's coefficient of similarity. Health status (*D*: Diseased, *H*: Healthy) and clone (*HOT*: Hotel, *CHA*: Charlie, *IND*: India, *JUL*: Juliet, *SV1*, *SX61* and *SX64*).

this genus in plant health (Grunig *et al.* 2008; Jumpponen and Trappe 1998). The results of this work suggest that *Phialocephala* may be pathogenic for both *Juliet* and *SVI* clones given its abundance in diseased plants. In contrast, *Chaetomium* sp. and *Rhizopus* sp. could be saprophytes or probably be related with some beneficial effects as indicated for their increase in abundance in healthy plants of these two clones.

The similarity observed among the fungal communities of the *SX64* and *SX61* clones is interesting given that among all the clones evaluated in this work only these two come from species that were recently introduced in North America. *SX61* (*S. sachalinensis*) is a shrub originally introduced from Japan while *SX64* (*S. miyabeana*), also a shrub, was introduced from east Siberia and China (Kopp 2001). In trials from the United States these two clones were observed to produce considerable amounts of biomass. In the present work, the isolation of the fungal communities associated with these two clones showed that *SX64* was preferentially associated with potentially beneficial fungi whereas *SX61* was associated mostly with potentially pathogenic fungi. This could be, therefore, a factor worth considering as part of the characteristics that contributed to the ability of *SX64* to produce higher amounts of biomass than *SX61* in plantations from eastern Canada (Labrecque and Teodorescu 2005).

The fungal communities associated with roots of the *Charlie* clone were characterized by a high fungal diversity in diseased plants and a low diversity in healthy plants. It is interesting that on the contrary to the other six clones, healthy plants of *Charlie* had a higher dominance of *Fusarium* sp. than diseased plants. Moreover, diseased plants were also associated with *Alternaria* sp., *Phomopsis* sp., *Hypocrea* sp. and *Penicillium* sp., which are genera that exhibited potential to be beneficial to the other clones. Little is known about the origin of the *Charlie* clone and no records exist about its survival in SRIC or about its capacity for biomass production.

Finally, the lack of statistical significance in the canonical analyses of both studies could be probably the result of the high diversity of taxa and the majority of them having a low abundance. However, it was evident that there are differences in regard to specific fungal functional groups, i.e. potentially beneficial and potentially pathogenic. Therefore, it could be expected that in studies that focus on specific taxa, rather than including the total diversity, the variables used in this study could be statistically relevant to explain the observed patterns.

4. EFFECTS OF POTENTIALLY BENEFICIAL AND PATHOGENIC FUNGI IN ROOTS OF WILLOW (*SALIX PURPUREA*)

4.1. Abstract

Willow short rotation intensive cultures could profit from the function of native beneficial fungal communities. Fungal isolates obtained from roots of the *Hotel* clone (*Salix purpurea* L.) were tested both *in vitro* and *in vivo* to evaluate their potential either as pathogenic or beneficial agents. *Fusarium culmorum*, *Didymella bryoniae*, and *Neonectria radicola* were selected out of nine potentially pathogenic fungi to be tested in combination with six potentially beneficial fungi. In the experiments *in vitro*, *Trichoderma harzianum*/*Hypocrea lixii*, and *Penicillium* sp. exhibited the highest antagonistic activity. The results from the direct inoculation of the fungi on willow cuttings did not result in significant differences on the overall aboveground biomass production. However, a negative effect was observed in photosynthetic activity and root development of some of the treatments inoculated with *H. lixii*, *Epicoccum nigrum* and *Umbelopsis ramanniana*. The results suggest that the *Hotel* clone is not susceptible to common phytopathogens found in agricultural soils but can be negatively affected by the presence of *Trichoderma*/*Hypocrea* sp. or by saprotrophs acting opportunistically.

4.2. Introduction

The energy derived from willow short rotation intensive crops (SRIC) is expected to be a sustainable alternative to fossil fuels, given that the overall production process, as well as the use of resources is judicious and non-harmful to the environment (Volk *et al.* 2004). Among the desirable characteristics of these sustainable systems, is the decrease in the use of pesticides and other chemical supplies of common use in current agricultural systems. This would not only position willow SRIC as a better alternative to fuels from non-renewable resources but could also facilitate the adoption of the biomass technology by government and general public.

Diseases are among the most important threats for agricultural systems. *Salix* species are susceptible to a number of stem and leaves diseases caused by fungi (Royle and Ostry 1995; Spiers 1998), but so far no root pathogens have been found to be a major risk for biomass crops. However, it was previously found that willows established in agricultural soils harbor a high diversity of fungi, some of which are well-known pathogens of other agriculture and forestry

crops (see Chapter 3). Moreover, willows grown under SRIC could be more susceptible to diseases than in natural forests, due to the particular characteristics of monoculture and clonal systems of these plantations (Royle and Ostry 1995), and to the range of adaptability that some pathogens can exhibit.

On the other hand, soil biota associated with willow roots can also be a rich source of natural biocontrol and biofertilizer organisms (Chatli *et al.* 2008; Van der Heijden 2001). Therefore, soil microorganisms represent an asset that could be used in benefit of the sustainability of SRIC. Studies have demonstrated, for example, that root endophytic bacteria have the ability to increase plant growth and development in poplars (*Populus* spp.) (Van der Lelie *et al.* 2009) and fungal inoculation has proved to promote the growth of certain *Salix* species (Adams *et al.* 2007; Van der Heijden 2001).

Common saprophytic fungi in temperate forests include among others *Penicillium* sp., *Hypocrea/Trichoderma* sp., *Umbelopsis* sp. and *Bionectria* sp. Species included in these genera have been used as biocontrol agents of pathogenic fungi as well as of other groups of organisms (Chaverri and Samuels 2003; Fang and Tsao 1995; Schroers 2001), and even some of their enzymes have been studied for their use in biocontrol and in decomposition of organic material (Baum and Hryniewicz 2006; Harman 2006).

Since willow SRIC are mainly intended for biomass production, the activity of potentially beneficial fungi would ideally promote plant growth. This promotion could occur in a direct way or through indirect effects such as the induction of resistance to pathogen attack and facilitation of the establishment and development of cuttings, among others (Baum and Hryniewicz 2006; Chatli *et al.* 2008). Since in some cases the beneficial effects can be indirect; an approach that includes antagonistic fungal functional groups found in SRIC may be useful to obtain accurate conclusions regarding the effects of potentially beneficial fungi on plant growth.

4.3. Objectives and Hypothesis

The hypothesis underlying this study was that the inoculation of *Salix* cuttings with potentially beneficial fungi can overpower the negative effects, in reference to cutting development, caused by potentially pathogenic fungi isolated from SRIC systems. The objectives of this study were to: *i*) test the pathogenic potential of fungi isolated from roots of diseased willows, *ii*) test the antagonistic ability of potentially beneficial fungi against potential

pathogens, and *iii*) assess the effects of the inoculation of potentially pathogenic and potentially beneficial fungi on the development of cuttings of the *Hotel* clone (*Salix purpurea* L.).

4.4. Materials and Methods

4.4.1. Pathogenicity Potential

Nine potentially pathogenic fungi, that had been isolated from roots of diseased willows, were selected. The chosen isolates were *Fusarium culmorum* (OTU 180), *F. redolens* (OTU 250), *F. oxysporum* (OTU 260), *F. equiseti* (OTU 350), *Truncatella angustata* (OTU 320), *Phomopsis columnaris* (OTU 410), *Didymella bryoniae* (OTU 450), *Neonectria radiculicola* (OTU 520) and *Penicillium* sp. (OTU 870).

An apple-based *in vitro* test was used to compare the pathogenicity of the isolates according to the protocol proposed by De Lange *et al.* (1998) for pathogens of woody species. Briefly, subcultures of the original isolates were made in PDA media following standard microbiological procedures. These subcultures were left to grow for five days in darkness. Healthy mature Golden Delicious apples (*Malus domestica* Borkh.) were surface sterilized with ethanol (95%) and a cubic piece of tissue (1 cm³) was removed using a sterile scalpel. A square (1 cm²) of mycelium was then extracted from the edge of the fungal culture and placed inside the apple. Finally, the cubic piece of apple was inserted back in its original position. Apples were then incubated in humid chambers and in darkness for two weeks when the development of infection was evident. At this point the diameter of the lesion caused by the fungi was measured. Controls were inoculated with a square of sterile PDA. Five repetitions were performed for each potentially pathogenic fungus.

4.4.2. Antagonistic Potential

Three potentially pathogenic fungi (*F. culmorum* (OTU 180), *D. bryoniae* (OTU 450), and *N. radiculicola* (OTU 520)) were selected based on two criteria: *i*) isolation frequency from roots of diseased plants belonging to the *Hotel* clone and *ii*) the response induced in the experiment with Golden Delicious apples. These fungi were co-cultured with six potentially beneficial fungi, for instance: *Epicoccum nigrum* (OTU 1070), *Dothidotthia aspera* (OTU 210), *Hypocrea lixii* (OTU 670), *Penicillium* sp. (OTU 870), *Umbelopsis ramanniana* (OTU 660) and *Bionectria ochroleuca* (OTU 990). After six days the diameter of the pathogen's mycelium was

measured. In cases where the mycelium was not completely circular, the diameter was measured in two directions at 90° to each other and the average of the two measures was used for the calculations. Percentage inhibition was calculated as $(a-b)*100/a$. Where a is the radius of the pathogen growing alone and b is the radius of the pathogen growing in co-culture with the potentially beneficial fungi. The experiment was repeated three times for each combination of potentially pathogenic with potentially beneficial fungus.

4.4.3. Plant Material for Greenhouse Test

Healthy stems of *Hotel* clone were collected in October 2009, from plants growing in the plantation belonging to the University of Saskatchewan (UTM 12U E0389931.8, N5776381). The stems were trimmed to 15 cm and surface sterilized by submerging in 2% H₂O₂ for one minute, and rinsed with sterile distilled water. After sterilization the cuttings were rooted in tap water for three weeks. Equally developed cuttings were selected for the test.

4.4.4. Inoculation of Cuttings and Experimental Design

Three potentially pathogenic fungi (*F. culmorum* (OTU 180), *Didymella bryoniae* (OTU 450), and *N. radicola* (OTU 520)) and six potentially beneficial fungi (*E. nigrum* (OTU 1070), *Dothidotthia aspera* (OTU 210), *H. lixii* (OTU 670), *Penicillium* sp. (OTU 870), *U. ramanniana* (OTU 660) and *B. ochroleuca* (OTU 990)) were tested. Each fungus was grown in PD broth (BD and Co.) for three to seven days, depending on their distinct rapidity of growth, until obtaining a suspension of mycelium-producing units (10⁴ units mL⁻¹).

Equally developed cuttings were randomly selected for the test. Rooted cuttings were first weighted and then root-dipped in the suspension of the potentially beneficial fungus for two hours. After the inoculation with the potentially beneficial fungus, cuttings were transferred to pots containing sterilized substrate (Sunshine Mix #4). The inoculation of the potentially pathogenic fungus was performed 48 h after the transplanting. In order to avoid further disturbance of the root system during the inoculation of the potentially pathogenic fungus, two sterile centrifuge tubes (15 mL) had been placed during the transplanting next to the cuttings and at the same depth as the roots (see Appendix) (Dhingra and Sinclair 1995). For the inoculation of the potentially pathogenic fungus the centrifuge tubes were removed and each well was filled

with 10 mL of the fungal suspension. After the suspension was added the wells were covered with substrate.

The control treatments were inoculated following the same procedure described above, but replacing the fungal suspensions with sterile PD broth.

The experimental design for this essay included 28 treatments which resulted from the combination of two variables and their respective levels: potentially pathogenic fungi (*F. culmorum*, *Didymella bryoniae*, *N. radicola* and non-inoculated) and potentially beneficial fungi (*E. nigrum*, *Dothidotthia aspera*, *H. lixii*, *Penicillium* sp., *U. ramanniana*, *B. ochroleuca* and non-inoculated). Three repetitions were performed for each treatment.

4.4.5. Harvesting

Plants were grown for eight weeks under greenhouse conditions (daytime temperature: 22°C, night time temperature: 20°C and 13 h of light). The pots were watered three times per week. At harvesting the height of the plants was measured and the photosynthetic activity of the plant was determined using a chlorophyll fluorometer (OS-30p, Opti-Sciences, Inc., USA). Fluorescence readings were taken in the third most recently developed leaf. Since most of the cuttings developed multiple stems, two readings were made, each in a different stem, in order to obtain an average value per experimental unit.

Plant biomass was separated into leaves, stems and roots and fresh and dry weights were determined for each part. Biomass production was calculated as the sum of the weights of fresh leaves and fresh stem minus the weight of each cutting at the beginning of the experiment. Root systems were recovered by digging the entire volume of substrate. Roots were collected in fine sieves, rinsed with tap water to clean off the excessive soil and tapped with absorbent tissue paper to measure the fresh weight (Adams *et al.* 2007). Dry weight was recorded after each sample was dried for 48 h at 70°C in oven.

4.4.6. Data Analyses

Data from each experiment were analyzed with Kruskal-Wallis tests. Multiple comparisons were carried out with the Conover-Inman test available in the software SYSTAT 13 (SYSTAT Software, Inc.).

4.5. Results

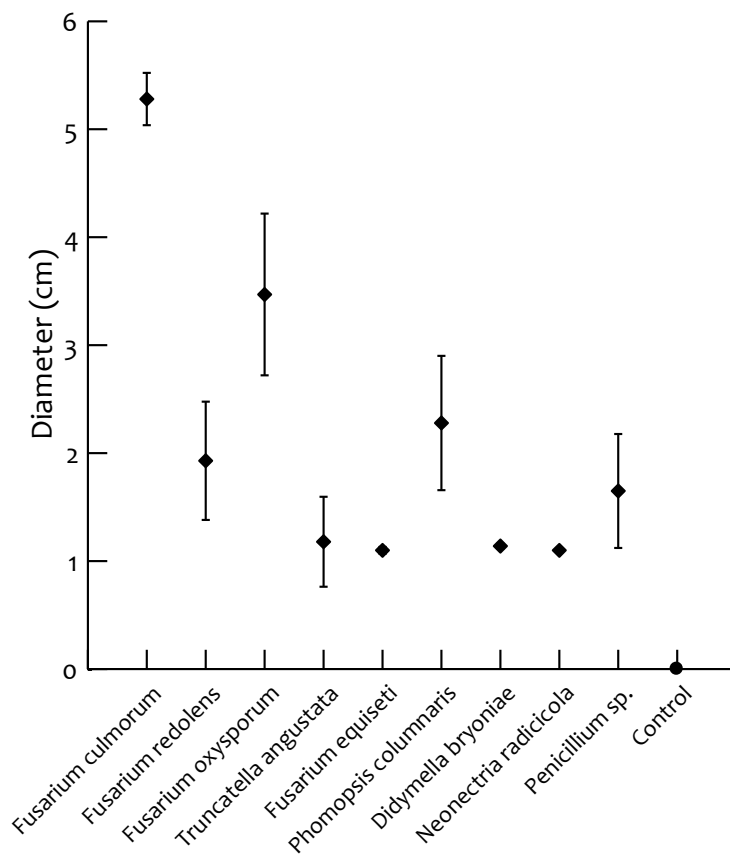
The potentially pathogenic fungi inoculated in the apples were able to induce some level of necrotic damage compared to the controls and statistical differences between treatments were observed ($P < 0.05$) (Figure 4-1). Apples inoculated with *F. culmorum* developed lesions significantly larger than most of the other potentially pathogenic fungi tested (Figure 4-1). On the other hand, *F. oxysporum* and *P. columnaris* produced a medium necrotic damage whereas for *F. equiseti*, *N. radicicola*, *Didymella bryoniae* and *T. angustata* the response was low.

The antagonistic relationship between potentially pathogenic and potentially beneficial fungi was studied with co-cultures. For all three potentially pathogenic fungi, antagonistic relationships were observed with at least one of the potentially beneficial fungi (Figure 4-2). In general, *H. lixii* showed the highest antagonistic ability against all three potential pathogens. In addition to *H. lixii*, *Penicillium* sp. also exhibited a high activity principally against *N. radicicola* and *D. bryoniae*. Regarding *F. culmorum*, both *Dothidotthia aspera* and *H. lixii* exhibited the highest antagonistic activity.

Overall, the inoculation of the willow cuttings with potentially beneficial and potentially pathogenic fungi did not produced major effects for most of the variables measured (Figure 4-3). Regarding total biomass production, the inoculation with potentially pathogenic fungi did not appear to affect the development of the plants compared to the controls. A few treatments presented a positive effect on biomass production, and the most notable was on plants inoculated with *Dothidotthia aspera* together with *N. radicicola*. Negative effects were observed on plants inoculated with *Penicillium* sp. together with *Didymella bryoniae*., and plants inoculated with *H. lixii* together with *D. bryoniae* (Figure 4-3a). In these cases, however, the negative effect appeared to be related to the presence of the potentially beneficial fungi rather than the potentially pathogenic.

The most significant effects of the treatments on the production of fresh aboveground biomass were observed on leaves rather than on stem (Figure 4-3). Thus, for example, plants inoculated with *N. radicicola* combined with either *E. nigrum*, *Dothidotthia aspera* or *B. ochroleuca* produced significantly more fresh leaves biomass than the rest of the treatments. On the other hand an increase on fresh stem biomass was only significant for plants inoculated with *Dothidotthia aspera* and *N. radicicola*. The negative effect of the inoculation with *U. ramanniana* appeared to affect fresh biomass of leaves and stem; however, it was not evident

a



b



Figure 4-1. Test of potential pathogenicity of fungi isolated from roots of *Salix* spp. **a)** Diameter of the lesions caused by potentially pathogenic fungi (*F. culmorum*, *F. redolens*, *F. oxysporum*, *T. angustata*, *F. equiseti*, *P. columnaris*, *D. bryoniae*, *N. radicola* and *Penicillium* sp.) in inoculated Golden Delicious apples. Bars indicate standard errors **b)** Lesions observed in apples two weeks after the inoculation with *F. culmorum*.

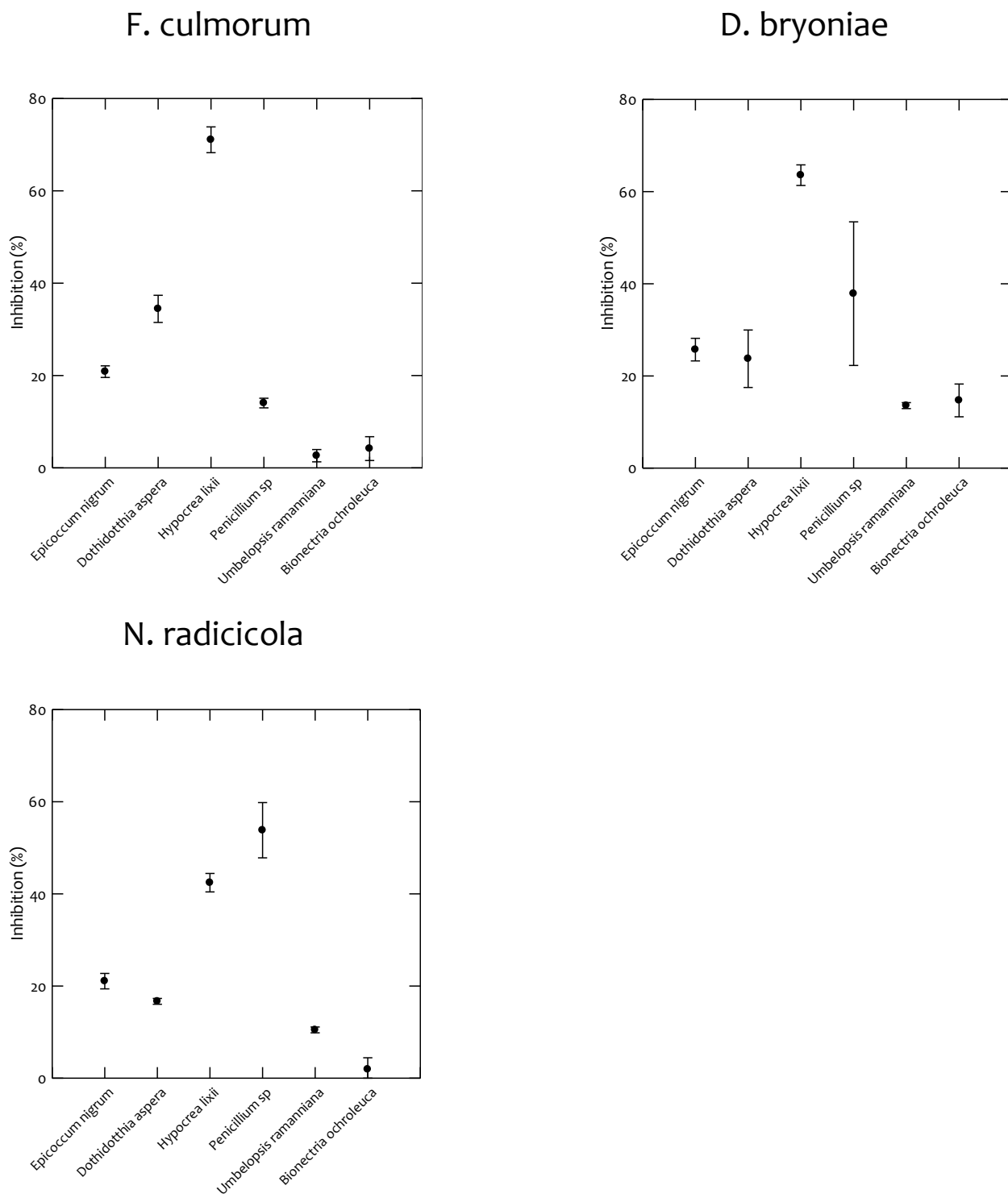


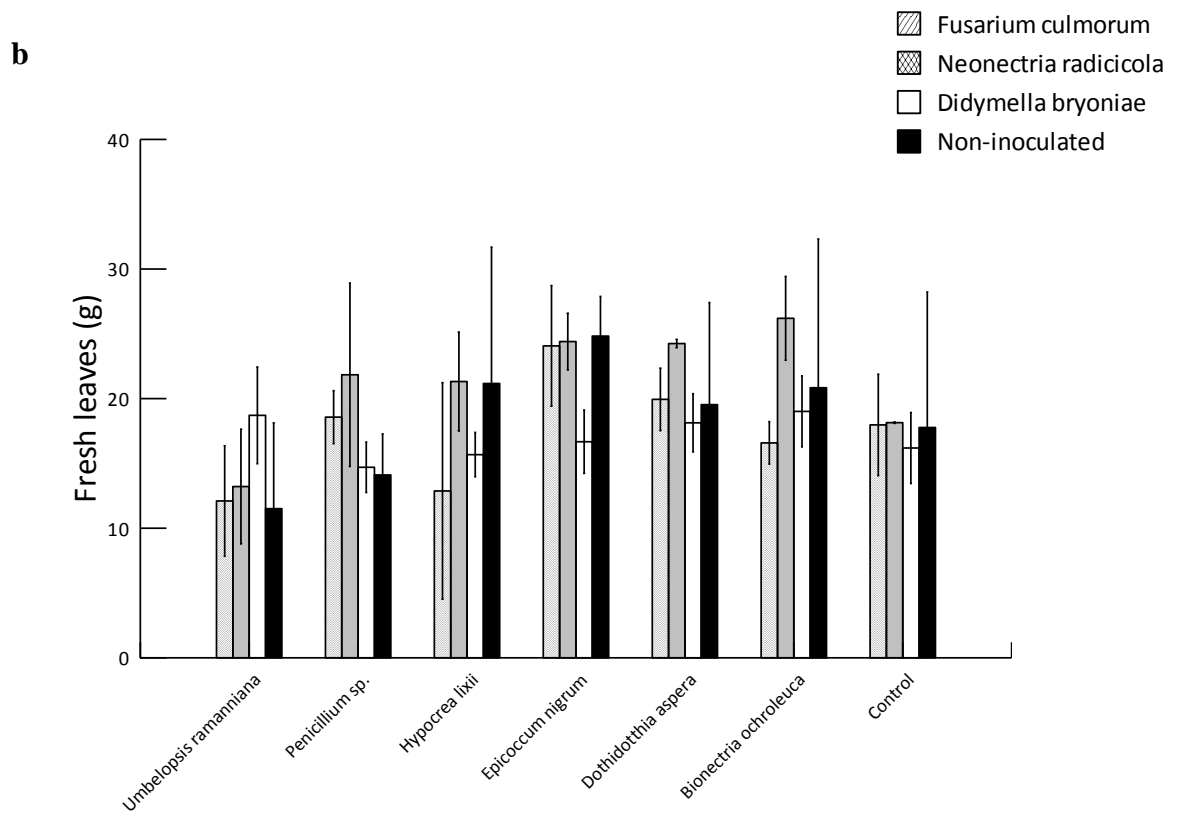
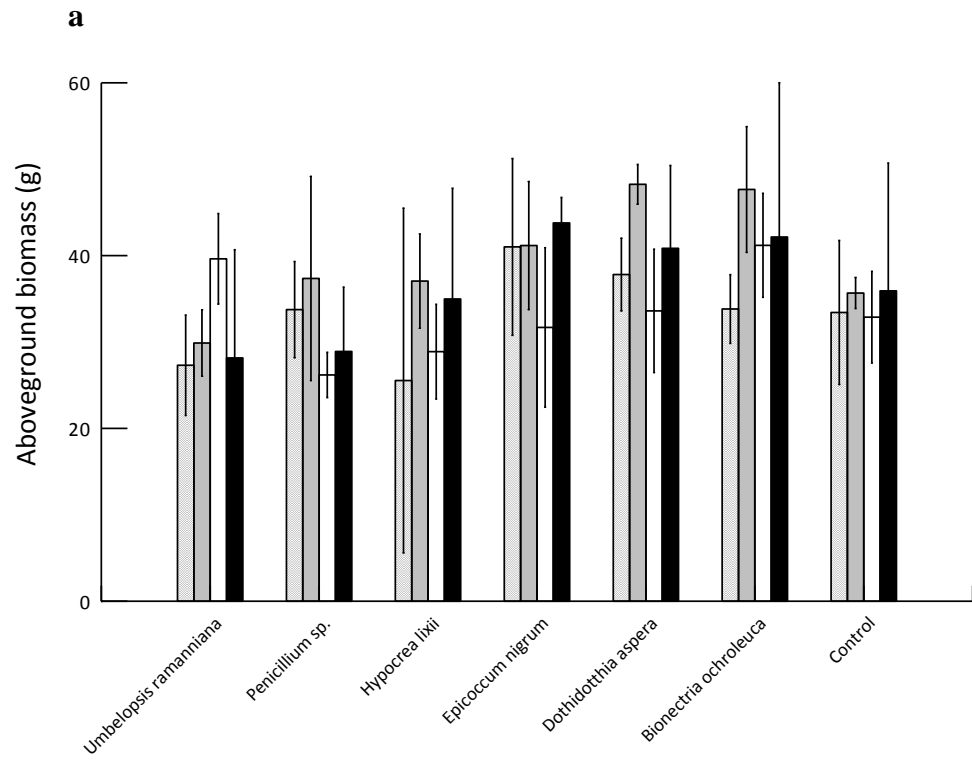
Figure 4-2. Antagonistic relationship observed in co-cultures of potentially beneficial (*E. nigrum*, *D. aspera*, *H. lixii*, *Penicillium* sp., *U. ramanniana* and *B. ochroleuca*) and potentially pathogenic fungi (*F. culmorum*, *Didymella bryoniae*, and *N. radicicola*).

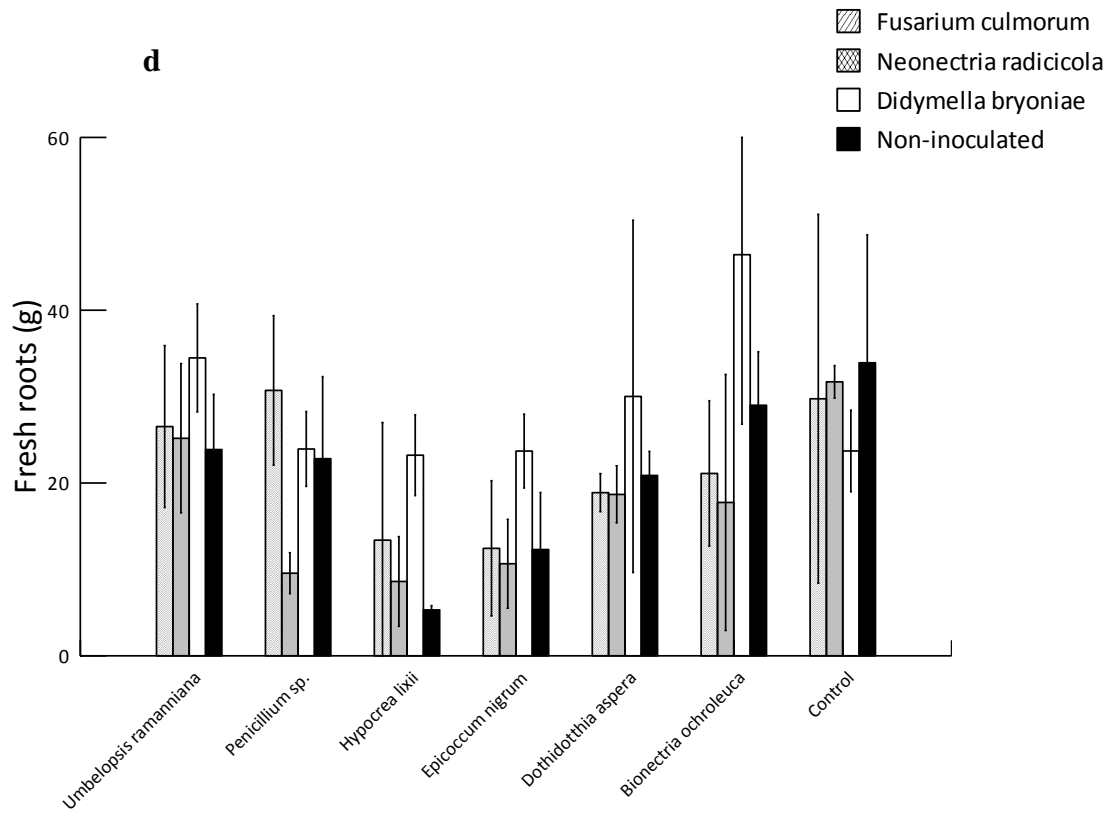
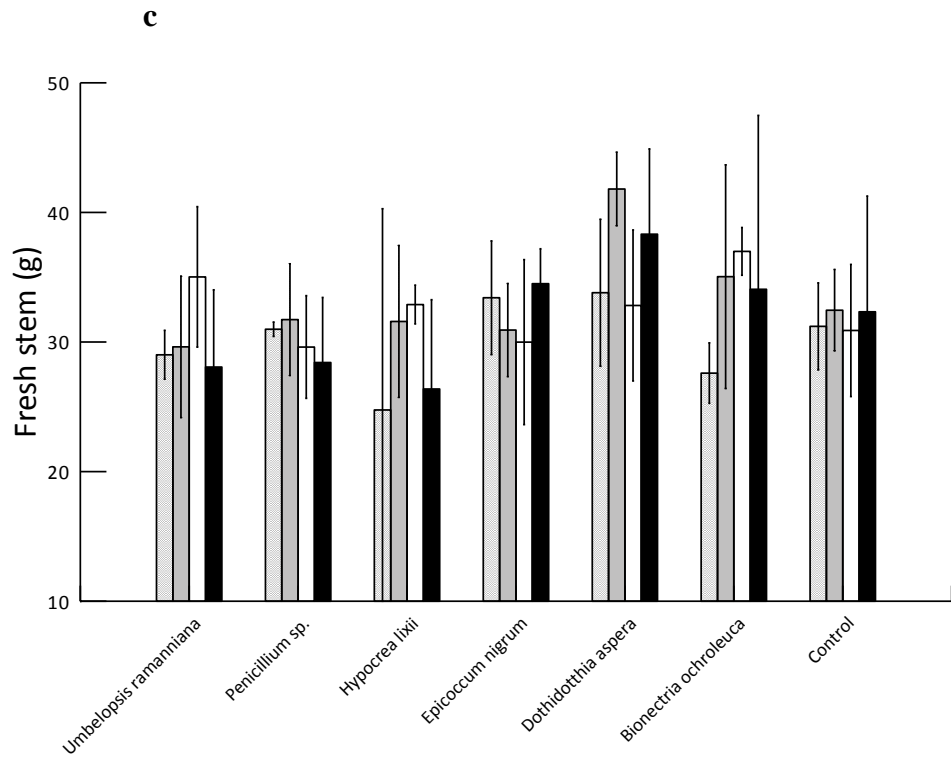
in the data from dry weight. A negative effect of the inoculation of potentially pathogenic fungi was observed on the stem dry biomass compared to the control plants. At the same time, the general positive effect of the inoculation with *Dothidotthia aspera* was more clearly evidenced in the dry biomass readings.

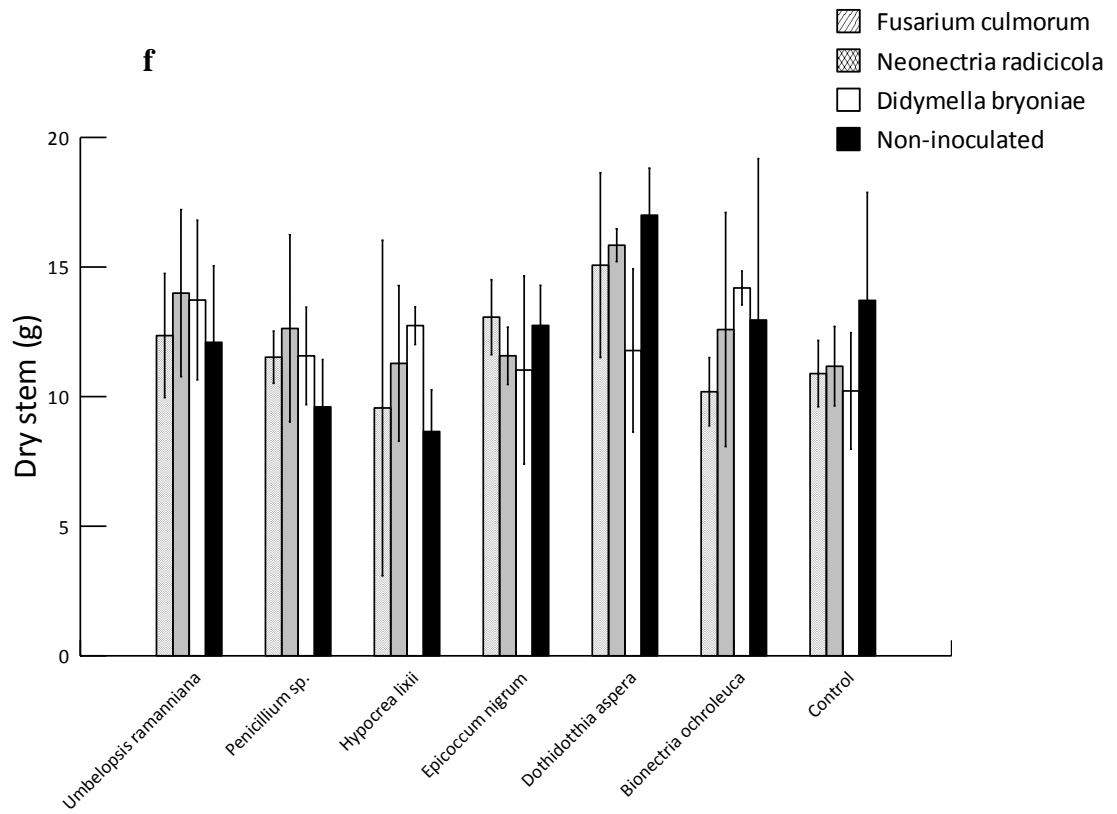
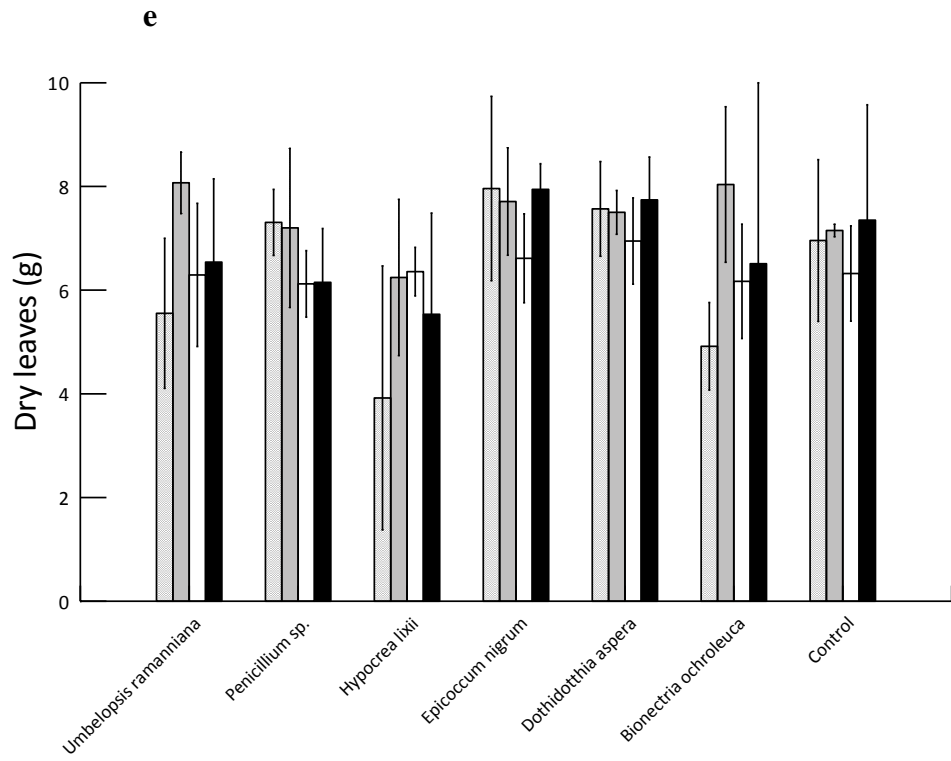
The effects of the inoculation of potentially pathogenic and potentially beneficial fungi were more noticeable with respect to root biomass than with respect to the aerial parts (Figure 4-3). The inoculation of pathogens produced a general negative effect compared to the controls, and such differences were more accentuated when observing the weight of dried roots. The inoculation of potentially beneficial fungi had also a negative effect on root development, except for plants inoculated with *B. ochroleuca* together with *Didymella bryoniae*, which produced more root biomass than the other treatments. The inoculation with *H. lixii* as well as the inoculation with *E. nigrum* had a significant detrimental effect on fresh and dry root biomass. Contrary to its negative effects on aboveground biomass production, *U. ramanniana* did not affect significantly root development and the root biomass of treatments that included this fungus was similar to the biomass of the controls.

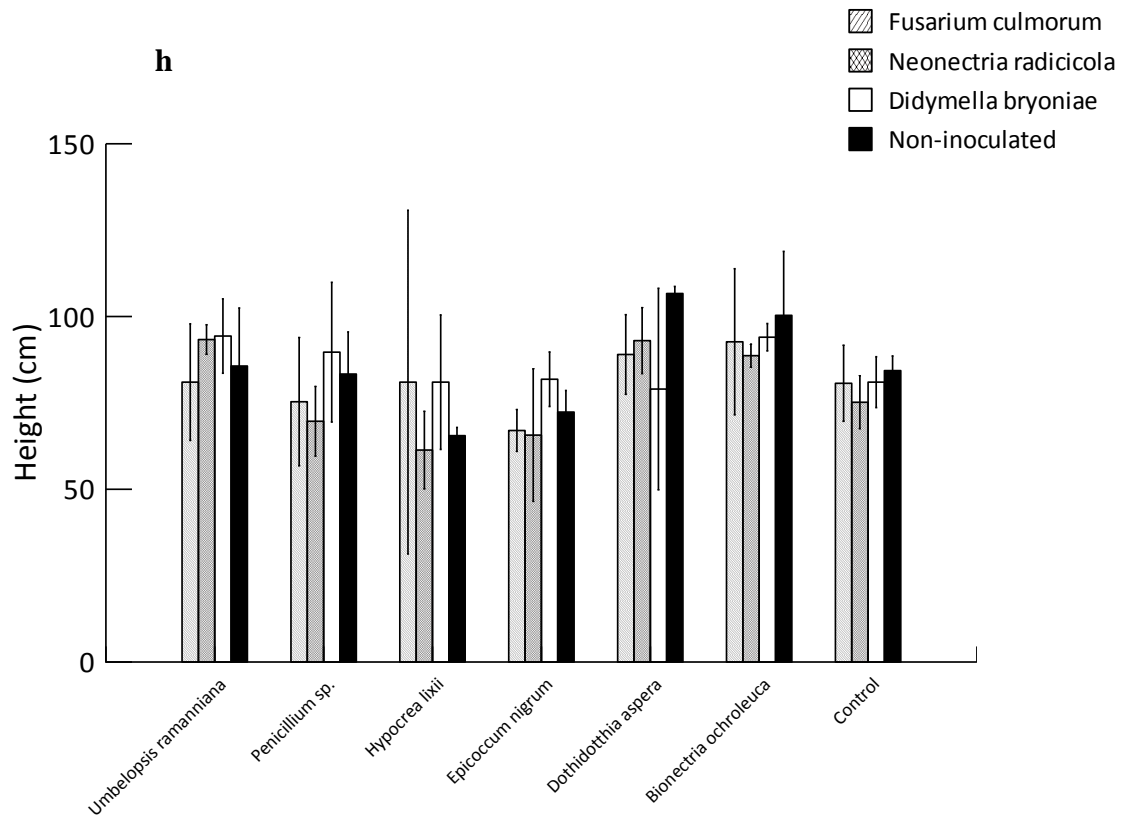
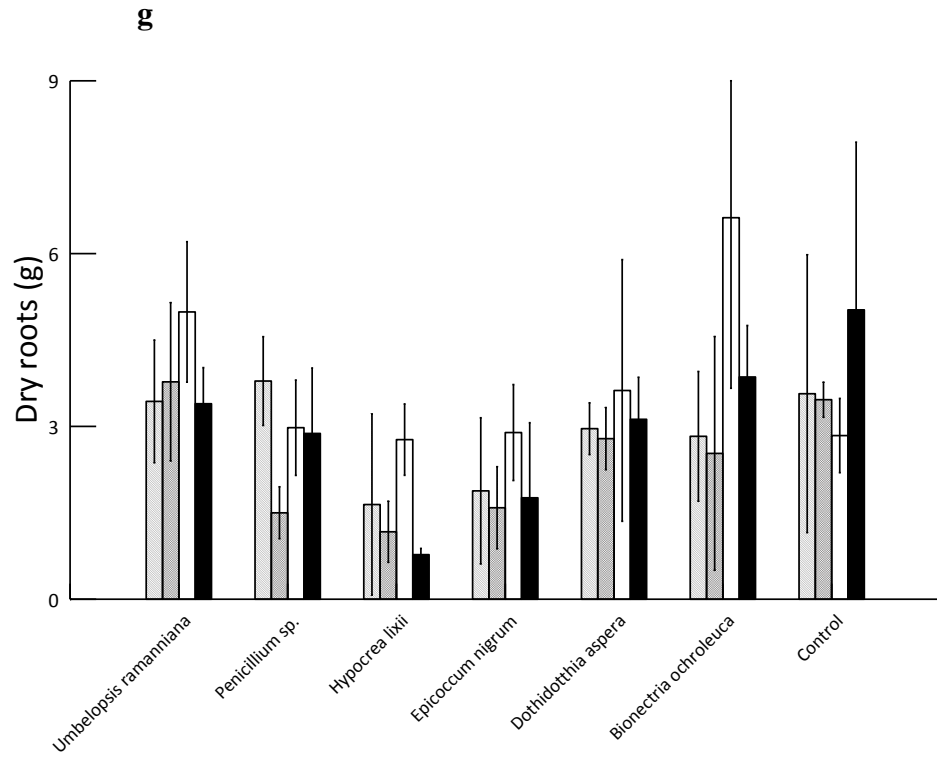
Regarding photosynthetic activity and growth in terms of height, all treatments produced very similar results (Figure 4-3). However, plants inoculated with *Dothidotthia aspera* alone, grew taller than the rest of the treatments, although when inoculated together with any of the potentially pathogenic fungi, the increase in height was less pronounced. On the other hand, plants inoculated with *E. nigrum* and *H. lixii* were shorter compared to the other treatments except when they were inoculated in combination with *Didymella bryoniae*.

Lastly, the measurements of photosynthetic activity were also comparable between treatments with exception of plants inoculated with *U. ramanniana*, which exhibited lower activity than the rest of the treatments. On the contrary, plants inoculated with *Penicillium* sp. presented a relatively high photosynthetic activity except when inoculated together with *Neonectria radicicola*. High values of photosynthetic activity were also observed in the treatments combining *H. lixii* and *Didymella bryoniae* as well as in the treatment combining *E. nigrum* and *Didymella bryoniae*.









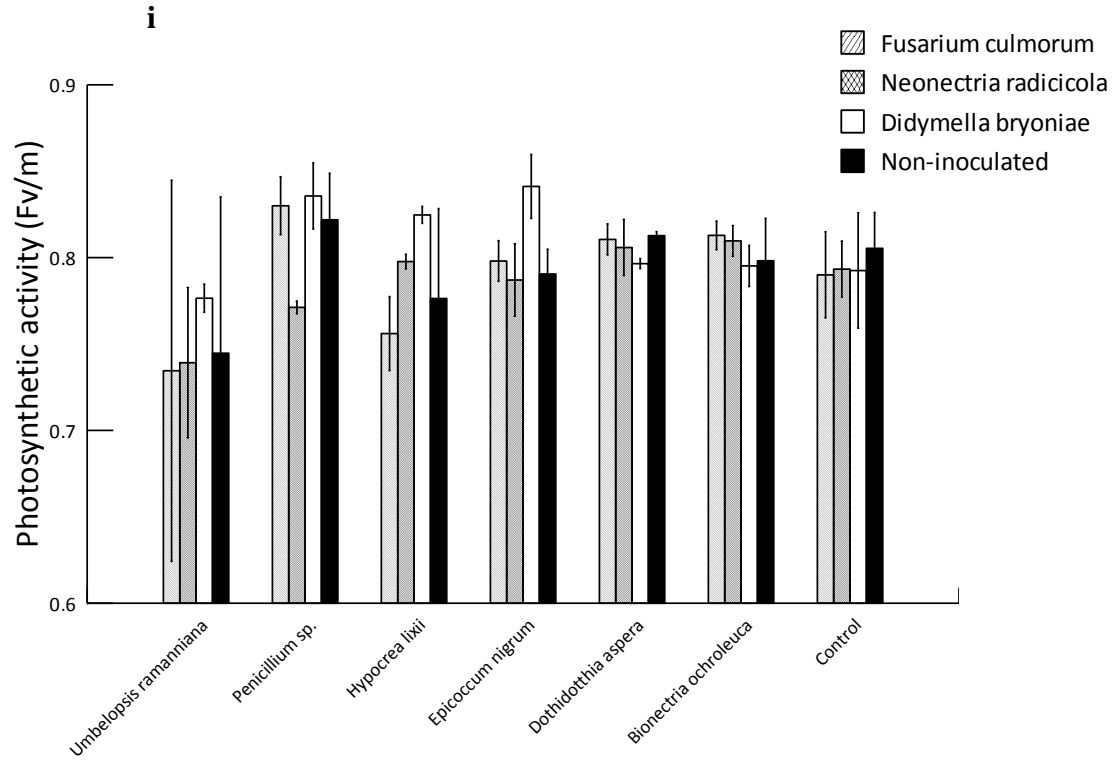


Figure 4-3. Effect of the inoculation of potentially beneficial (*E. nigrum*, *Dothidotthia aspera*, *H. lixii*, *U. ramanniana* and *B. ochroleuca*) and potentially pathogenic fungi (*F. culmorum*, *N. radicola* and *Didymella bryoniae*) on *Hotel* clone cuttings **a**) Aboveground biomass **b**) Fresh leaves biomass **c**) Fresh stem biomass **d**) Fresh roots biomass **e**) Dry leaves biomass **f**) Dry stem biomass **g**) Dry roots biomass **h**) Height **i**) Photosynthetic activity. Bars correspond to standard errors.

4.6. Discussion

The parameters to decide whether a potential pathogenic fungal species should be considered or not a real threat to a crop are numerous and highly dependent on the system of study (Agrios 2005). Without doubt, however, the most accurate criteria are given only by the actual devastating effects that are observed once it is too late to prevent them. Therefore, it is important to recognize latent risks that may eventually become problematic for a crop. Poplar bioenergy crops have already been seriously threatened by rust diseases for which farmers are not prepared to respond (Royle and Ostry 1995). This experience has warned of the importance of monitoring and managing the dynamics of fungal communities in willow SRIC in order to avoid the unexpected development of disease outbreaks.

The potentially pathogenic fungi tested here were all isolated from diseased *Salix* plants and when tested they were able to cause different degrees of damage to the apple tissue. The use of apples has been proposed as an effective and accurate system to pre-screen the pathogenic ability of fungi found in forestry species without the need to wait until growing and infecting actual trees (De Lange *et al.* 1998). In this test, *F. culmorum* stood out from the rest of the tested fungi due to its high capacity to induce damage on the apples. This fungus was found to be highly dominant in diseased and young plants of the *Hotel* clone growing under SRIC (see Chapter 3) but has not been reported as pathogen of *Salix* spp. Furthermore, the results from the greenhouse test did not suggest a particular negative effect of this fungal species on the development of willow cuttings, which contrasts with the high susceptibility typically observed in other crop systems such as in cereals (Obanor *et al.* 2010). However, it is important to acknowledge the genetic flexibility exhibited by members of *Fusarium* sp. and consider the effects that they may have on the development of willow cuttings under field conditions where the environment may be more favorable for these fungi.

The other two potentially pathogenic species that were chosen for the greenhouse experiment are also reported as pathogens of other cultivated and forestry plant species (Desjardins 2006; Kwasna and Bateman 2009) but under greenhouse conditions they did not cause serious damage to willow cuttings. This could be interpreted as an indication of the low susceptibility of willows (in this case of the *Hotel* clone in particular), to fungi that are normally found in agricultural soils. Moreover, it could support the general opinion that root-associated diseases do not present a high risk for the development of SRIC (Hubbes 1983), or at least not as

high as other diseases infecting stem and leaves. Nonetheless, the combination of multiple factors found under field conditions, such as the amount of inoculum in the soil, clone susceptibility, soil physicochemical characteristics, management practices and weather, will ultimately define whether or not these species could become a serious threat for willow plantations (Agrios 2005).

A desirable achievement in the development of sustainable willow plantations is the adequate use of the resources that are naturally available on site (Abrahamson *et al.* 1998), while minimizing the introduction of external agents into the ecosystem. The present work demonstrated that fungi isolated directly from roots of willows grown under SRIC have characteristics to antagonize potential pathogens that could affect willow plants. Species of *Hypocrea*, *Penicillium* and *Bionectria* have been previously recognized as antagonists of pathogenic fungi of traditional crops (Chaverri and Samuels 2003; Fang and Tsao 1995; Schroers 2001) but their mechanisms of antagonism have not been explored except for some species of *Trichoderma/Hypocrea*. In fact, the available studies regarding the other two genera seem to be focused on the ability of some species of *Penicillium* to solubilize phosphorus (Baum and Hryniewicz 2006; Chatli *et al.* 2008) rather than in its ability to control pathogens. In the case of *Bionectria* sp., it is generally its unspecific saprotrophic ability that is recognized, and only a few species have been tested as potential biocontrol agents (Schroers 2001).

An apparent lack of response, in terms of aboveground biomass production, was observed after the inoculation with potentially beneficial fungi compared to non-inoculated plants or to plants inoculated with potentially pathogenic fungi. The inoculation with *Dothidotthia aspera* however, appeared to have a particularly positive effect on stem biomass. Further experiments may be useful to clarify whether it holds a relevant aptitude to stimulate stem biomass production in *Salix* spp. as well as to clarify its taxonomical affiliation.

In spite of the absence of effect on aboveground biomass production observed in the majority of treatments, plants inoculated with *H. lixii* exhibited a significant negative response in root biomass and height. Previously, Adams *et al.* (2007) reported a positive effect in the development of cuttings of *Salix fragilis* L. or crack willow, after inoculating *T. harzianum* T22 (*H. lixii*). Thus, the results of the present study provide evidence of the variability of the benefits that are commonly attributed to *Trichoderma/Hypocrea* species. Furthermore, the fact that the *H. lixii* isolate used in this work was originally obtained from diseased willows growing under

SRIC may be an additional proof of its potential negative effect under field conditions. This finding is relevant also because currently *Trichoderma*-based products are widely used in agriculture systems. The aim of such products has been to replace or at least reduce the amount of chemically synthesized compounds used to control diseases or to stimulate plant growth (Harman 2006). However, the survival and dispersion of *Trichoderma/Hypocrea* sp. on soil after the application of these products have not been studied until recently (Longa *et al.* 2009). Since the traits of *T. harzianum/H. lixii* usually are strain-specific, it may be important to acknowledge the potential implications of the application of *Trichoderma/Hypocrea* sp. products and their resilience in current arable fields which eventually will be replaced by willow SRIC.

Among the species of root-associated fungal communities, *Umbelopsis ramanniana* has been frequently isolated from forest soils of the northern hemisphere. Although the current research on this species has been focused on the production of enzymes involved in lipid synthesis (Lardizabal *et al.* 2008); previously its ecological ubiquity had led researchers to investigate its role in plant establishment and development in forestry species (Summerbell 2005). In this work, plants inoculated with *U. ramanniana*, exhibited a low photosynthetic activity and a tendency to decrease the production of the aboveground biomass compared to the other treatments. Previous works also reported negative effects on the development of woody plants when inoculated with this fungus (Summerbell 2005); however the damage caused by *U. ramanniana* is less severe than the damage that would be expected from root rots or damping-off. Therefore, it could be assumed that its function is principally saprotrophic but may be harmful in cases where the plant has already been attacked by a pathogen.

It has been hypothesized that the function of saprotrophs may benefit plant growth through the transformations that their metabolic activities induce in the environment. Their benefits can be also the result of the control of pathogenic agents or, in some cases, a direct influence on plant metabolism (Trillas and Segarra 2009). For example, some saprotrophs could induce the production of root tannins which are a plant defense against pathogen attack (Summerbell 2005). Also, in the specific case of *Salix* spp. the enzymatic activity of some saprotrophs has been associated with benefits on plant nutrition (Baum and Hryniewicz 2006). However, it is not clear whether saprotrophic fungi that exhibit a high antagonistic but unspecific activity, such as *Hypocrea* sp., *Penicillium* sp. and *Epicoccum* sp. could indirectly affect willow growth by inhibiting beneficial endophytes such as mycorrhizas (Adams *et al.* 2007; Werner *et*

al. 2002). Therefore, it still remains to evaluate the effects of saprotrophic fungi in SRIC considering other types of benefits. For instance, not exclusively in terms of their ability to control pathogens but also, in terms of their ability to promote the association of willows with other fungi, their ability to liberate nutrients to be taken up by the plants, or their ability to confer resistance to climatic stresses.

5. PCR-DGGE OF ARBUSCULAR MYCORRHIZAL FUNGI IN WILLOW BIOMASS PLANTATIONS

5.1. Abstract

Among the endophytic fungi living in the roots of willows, arbuscular mycorrhizal fungi (AMF) stand out for their positive effects on plant growth, disease resistance and climatic adaptation. An approach based on the denaturing gradient gel electrophoresis (DGGE) technique was used here for the study of AMF communities in the roots of seven *Salix* clones. The PCR amplification with AMF specific primers was negative for clones *SX61* and *SX64* indicating that they do not associate with these fungi. The remaining five clones, on the contrary, were positive for the mycorrhizal PCR amplification although, in some cases, the presence of AMF was detected only in healthy but not in diseased plants, or vice versa. The majority of the bands excised from the gel were matched to published sequences of *Glomus claroideum*, *G. irregulare* (*G. intraradices*) and *G. mosseae*. The role of these AMF species appears to be dependent upon the clone to which they associate. This calls attention to consider the implications, for biomass production, of the specificity of the association between particular willow clones and AMF. Given its accuracy and ease of implementation, DGGE appears to be a suitable technique for complex ecological studies intended at the scale of plantations.

5.2. Introduction

Arbuscular mycorrhizal fungi (AMF) play an important role in the ecology and physiology of plant communities (Van der Heijden and Sanders 2002). Evidence of the numerous benefits that plants can obtain from the association with AMF has been given by several studies during the past decades. Such benefits include, among others, the increase of dry biomass production, disease resistance and tolerance to several abiotic stresses (Van der Heijden and Sanders 2002). Moreover, AMF could have a positive impact on forest systems through the promotion of carbon sequestration and sustainable land management (Rooney *et al.* 2009), in addition to a potential increment of biomass production. These benefits have fueled the interest of developing mycorrhizal inocula that can be used in agricultural systems to enhance plant

performance and yields (Gianinazzi 2002). In practice, however, the effects of AMF vary from system to system and greatly depend upon the particular conditions of each crop.

Willow short rotation intensive crops (SRIC) have been established throughout the Canadian Prairie provinces (Alberta, Saskatchewan and Manitoba) to evaluate their potential to produce biomass for bioenergy. In natural environments, willows are able to form associations with AMF as well as with ectomycorrhizal fungi (Beauchamp *et al.* 2006; Dhillon 1994; Fujimura *et al.* 2008), a trait that has been described only for few plant genera so far. In spite of their apparent influence on plant development, it is not yet understood the role that these fungal–plant associations may play as contributing factors to the success of willows SRIC for biomass production. However, evidence suggests that mycorrhizal associations may be fundamental in the development of certain species of *Salix*. Under controlled conditions plants of *S. repens* were able to sprout and grow significantly better when inoculated with AMF compared to non-inoculated controls (Van der Heijden 2001). As a result, AMF are of interest for the development of SRIC as they could potentially influence the overall performance and adaptability of willows in these systems.

Even though the importance of AMF for agriculture and plant ecology has been recognized, many gaps still remain unsolved in reference to their phylogeny, ecology and physiology (Redecker and Raab 2006). The study of AMF has been difficult since, unlike other fungi, AMF do not grow in artificial media. Therefore, the advancement in molecular biology has considerably facilitated their study. The development of the polymerase chain reaction (PCR) technology has favored studies based on nucleic acids and, as a result, most of the recent findings on AMF have been derived invariably from both DNA and RNA analyses (Redecker and Raab 2006). Although useful, these approaches are very sensitive and give only partial information of AMF communities (Anderson and Cairney 2004).

In order to increase the understanding of AMF, studies using environmental samples require techniques that allow handling many specimens at once and are easy to implement. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is a technique that has been used in numerous fungal ecology studies with optimal results (Kowalchuk *et al.* 2002; Liang *et al.* 2008; Ma *et al.* 2005). The advantages of DGGE are, among others, the possibility to obtain information of the taxonomy of the AMF species, and to estimate the diversity of those communities using the banding pattern of the gel (Fromin *et al.* 2002). These characteristics

appear to be very useful in the case of willow SRIC since only little is known about the mycorrhizal status of the plants in these systems, and there is no clarity in reference to the AMF species present. More notably, the effects that AMF communities may have on the performance of different clones of interest for the research program on *Salix* spp. have not been considered yet. Since one of the major interests of the biomass program is the selection of suitable clones (Kopp 2001), it would be important to understand better the relation existent between AMF and specific clones.

In reference to DGGE, the usefulness and specificity of most of the primers developed so far is contradictory according to the results of different studies (Douhan *et al.* 2005; Ma *et al.* 2005; Yergeau *et al.* 2006). Recently, Höppener-Ogawa *et al.* (2009) demonstrated the suitability of the primer pair FLR3/FLR4 to specifically monitor AMF communities in relation to the effect of mycophagous bacteria on *Plantago* sp. The outcome of that work was very satisfactory and supported the appropriateness of that molecular technique for AMF ecological studies.

5.3. Objectives and Hypothesis

It was hypothesized that differences in the AMF communities associated with the roots of different *Salix* genotypes could be monitored using a high throughput molecular fingerprinting technique. Therefore, the objectives of this study were to: *i*) develop a protocol for the use of the DGGE in studies of AMF communities in roots of *Salix* spp., *ii*) identify AMF species colonizing the roots of willows grown under SRIC, and *iii*) establish differences or similarities between seven willow clones in regard to their associated AMF communities in plantations established in the Canadian Prairies.

5.4. Materials and Methods

5.4.1. Plant Material and DNA Extraction

Root samples were collected in 2008 from healthy and diseased willow clones from plantations established in Alberta, Saskatchewan and Manitoba (Table 5-1). A soil core (capacity $5.03 \times 10^3 \text{ cm}^3$) was used to sample at 30cm from the base of the stem taking the first 20 cm of soil to ensure that the root system belonged to the selected plant. Plants of the *Hotel* clone were sampled in the three provinces. The other six clones (*Charlie*, *India*, *Juliet*, *SV1*, *SX61* and *SX64*)

were sampled only in Saskatchewan (Table 5-1). Three randomly chosen healthy plants as well as three diseased plants were sampled for each clone.

The roots were initially cleaned by shaking off the excessive soil. Previous to DNA extraction the samples underwent a sterilization protocol which consisted of immersion in sterile distilled water (SDW) for one minute followed by ethanol (95%) for 10 sec, SDW for 10 sec, bleach (5%) for one minute, SDW for one minute and a final rinse with SDW for one minute. The roots were air dried in a laminar flow chamber; 100 mg of primary, secondary and fine roots were used given the preference of AMF to colonize them (Van der Heijden and Sanders 2002). DNA extraction was performed using the QIAGEN Plant DNeasy Mini kit (Qiagen Inc., Valencia, CA, USA), with a final dilution of 50 µL. The extracted DNA was preserved at -80°C until further processing.

5.4.2. PCR Conditions

The nested PCR approach consisted of a first amplification with fungi-specific primers LR1 and FLR2 (Trouvelot *et al.* 1999; Van Tuinen *et al.* 1998). Next, primers FLR3-GC and FLR4 (Gollotte *et al.* 2004) were used to amplify a section of the rRNA large subunit, with an approximate size of 380 bp. A GC clamp was added in the 5' end of the FLR3 primer in order to obtain suitable products to run DGGE. The resultant primer was FLR3-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG TTG AAA GGG AAA CGA TTG AAG T-3').

The program for the first amplification consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 93°C for one minute, annealing at 60°C for one minute and extension at 72°C for one minute, and a final step of extension at 72°C for 10 min. For the nested PCR, the products corresponding to each treatment's repetitions were pooled and diluted 1:10 and 1:100. Two microliters of the diluted products were used as template for the second PCR. In the second PCR, primers FLR3-GC and FLR4 were used and the amplification program was the same as for primers LR1 and FLR2 except that the annealing temperature was raised to 63°C to reduce the probability of unspecific amplification.

Reactions were carried out in a final volume of 25 µL. The PCR mixture contained 10X Qiagen buffer (containing MgCl₂), 0.4 µM of each primer, 200 mM of each dNTP, and BSA (0.3 µg/µL) was added to reduce PCR inhibition. For the second reaction, using the

Table 5-1. Site characteristics of *Salix* spp. plantations where communities of arbuscular mycorrhizal fungi were studied.

Province	Coordinates	Texture	pH	Carbon (%)	Nitrogen (%)	Year of establishment	Mean annual precipitation (mm) [§]	Clone
Manitoba (MB)	UTM 14U 0559416, N5534076	Clay loam overlying sandy loam	8.1-8.5	1.6-4.7	0.25-0.35	2005	374	
Alberta (AB)	UTM 12U E0330943, N5921366	Clay overlaying sandy loam	8.1-8.5	2.3-3.9	0.19-0.28	2006	459.6	Hotel
	UTM 12U E0330943, N5921366	Clay overlaying sandy loam	8.1-8.5	2.3-3.9	0.19-0.28	2005	459.6	
Saskatchewan (SK)	UTM 12U E0389931.8, N5776381.7	Heavy clay	6.5-7.5	2.0-2.6	0.18-0.30	2006	348.3	Charlie, Hotel, India, SV1, Juliet, SX64, SX61

[§] Climate average for the last 30 years according to Environment Canada (2010).

mycorrhiza-specific primers, the mixture was the same as before except that no BSA was added. A negative control was included in every reaction and for the nested PCR the negative control of the first reaction was re-amplified. After each PCR, products were visualized in a 1% agarose gel electrophoresis and stained with ethidium bromide.

5.4.3. DGGE

A DGGE was run using the amplicons obtained with the primer pair FLR3-GC/FLR4. The gradient used was 20 to 55% denaturant, as suggested by Höppener-Ogawa *et al.* (2009), where 100% denaturant solution contains 7 M urea and 40% (v/v) formamide. As this gradient proved to be excessively wide, it was further narrowed down to 40-60%. The electrophoresis was run for 16 h at 60°C at 80 V.

The gel was stained with SYBR-Green (Invitrogen) and visualized by UV illumination. Digital images were captured for analysis. Bands of interest were excised with a sterile scalpel, crushed in 30 µL of 1X TE buffer and centrifuged for two minutes. One microliter of the supernatant was used for re-amplification with primers FLR3/FLR4. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and submitted for sequencing at the Plant Biotechnology Institute (Saskatoon, Saskatchewan). Similarity analyses were performed using the BLAST module available from NCBI (<http://www.ncbi.nlm.nih.gov>).

Sequences obtained from the bands and from the GenBank database were aligned with Clustal W included in MEGA 4.1 software (Tamura 2007). A phylogenetic tree was estimated with the Maximum Likelihood method available in TOPALi version 2.5 software (Milne 2004). The substitution model used was HKY (Hasegawa *et al.* 1985). Tree reliability was estimated by a bootstrap test with 100 repetitions.

5.4.4. Banding Pattern Analysis

The DGGE banding pattern was codified into a presence-absence matrix based on the migration behavior of the bands of each sample. The matrix was submitted to CANOCO (Windows version 4.5) (ter Braak and Smilauer 2002) and a principal component analysis (PCA) was performed to study the association between species presence and clone identity.

5.5. Results

5.5.1. PCR Amplification with FLR3/FLR4 Primers

The amplification with the primers LR1 and FLR2 produced bands for all the samples thus confirming the presence of fungi in the roots. For some samples, the bands were not evident or were very faint (Figure 5-1). In those cases the samples were amplified using a more diluted template (1:100) in order to decrease the chance of inhibition by some substances that could be more abundant in those samples than in the others.

The nested PCR was positive for the following samples belonging to the *Hotel* clone: diseased plants from Alberta (2005 and 2006), healthy plants from Alberta (2005), Saskatchewan and Manitoba (Figure 5-2). Regarding the other clones, positive amplification was observed only in the following samples: diseased *India*, healthy and diseased *Juliet*, diseased *SVI* and healthy *Charlie* (Figure 5-2). No bands were observed in any case for the *SX61* and *SX64* clones.

5.5.2. DGGE

The majority of the samples produced a multi-banding pattern except for the sample corresponding to healthy *Hotel* from Manitoba which presented a unique predominant band (Figure 5-3). In total, 15 predominant bands with different migration behaviour were identified and excised from the gel for re-amplification and sequencing. All these bands were matched to Glomeromycota sequences deposited in the GenBank database (Table 5-2). The similarity percentage reported by BLAST was lower than 95% for bands 11, 21, 22, 41, 55 and 73.

The phylogenetic tree revealed that the sequences obtained from the roots of the *Salix* clones could be divided into four major groups. Thus, bands 73, 31, 41, 71 and 72 appeared to be closely related to *Glomus claroideum*; bands 11, 12, 51 and 103 were related to *G. intraradices*; and bands 34 and 55 were related to *G. mosseae* whereas band 33 was positioned close to *G. cf. diaphanum*. Finally, three distinct bands: 21, 22 and 32 formed a separated group here designated as Glo-S (Figure 5-4).

Differences in the composition of the AMF communities were observed between clones. In general, the *Hotel* clone appeared to associate mainly with *G. intraradices* independently of

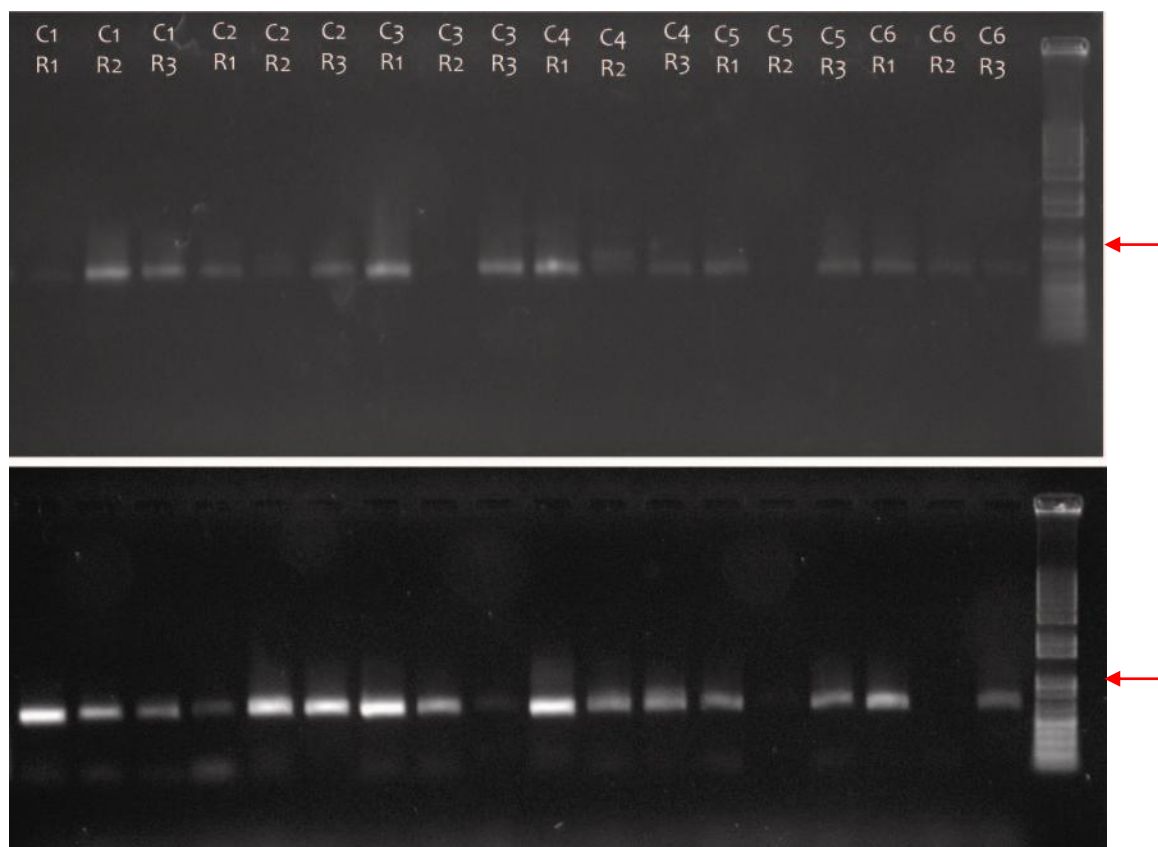


Figure 5-1. PCR amplification with the universal primers LR1 and FLR2 of diseased (top) and healthy (bottom) plants belonging to different willow clones. Showing the products of six clones (C1: *Charlie*; C2: *Hotel*; C3: *India*; C4: *Juliet*; C5: *SV1*; C6: *SX61*) and their corresponding three repetitions (R1-R3). Arrows indicate fragment size aprox. 850bp.

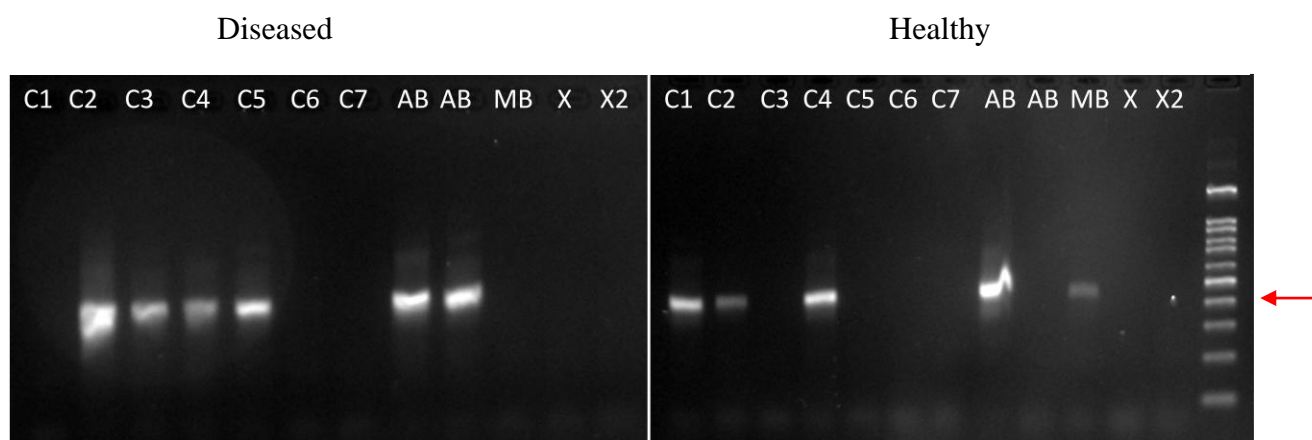


Figure 5-2. Products of the PCR amplification with mycorrhizal specific primers FLR3/FLR4 of diseased (left) and healthy (right) plants belonging to different willow clones (C1: *Charlie*; C2: *Hotel*; C3: *India*; C4: *Juliet*; C5: *SVI*; C6: *SX61*; C7: *SX64*). AB: Alberta; MB: Manitoba; X: negative control form first PCR; X2: negative control of the nested PCR. Arrow indicates fragment size aprox. 400 bp.

the province or the health status, although the samples of diseased *Hotel* from Manitoba and of healthy plants from Alberta (2006) were negative for the presence of AMF.

Diseased plants belonging to the *SVI* clone appeared to be colonized by *G. claroideum* while healthy plants of the same clone were negative for the presence of AMF. Similarly, only diseased plants of *India* were positive for AMF but in that case the bands appeared to form a separate clade which was denominated Glo-S (Figure 5-4). Plants belonging to the *Juliet* clone seemed to be able to associate with a variety of AMF since, bands from all four major groups were amplified from the samples of this clone. However, bands 31, 32 and 33 appeared to be associated with the diseased status of *Juliet* whereas bands 22 (Glo-S) and 34 (*G. mosseae*) were exclusively found in the healthy status. Regarding AMF in samples of *Charlie*, only healthy plants of this clone were positive for the PCR amplification and the bands recovered from the DGGE gel were all associated with the clade of *G. claroideum*.

5.6. Discussion

DGGE proved to be a useful tool for the study of AMF communities associated with willows grown under SRIC. Traditionally, studies dealing with AMF have not only been limited by the laboriousness of the isolation of spores and estimation of root colonization but most importantly due to the great difficulty of establishing the phylogenetic affiliation of the mycorrhizal fungi (Sanders *et al.* 1995; Schussler *et al.* 2001b). For example, in the study by Khasa and collaborators (2002) clear differences were observed regarding the levels of mycorrhizal colonization among 28 *Populus* clones grown under SRIC but no attempt was made to identify the species of AMF. In the present study, DGGE was satisfactorily used to identify the species of AMF associated with the roots of *Salix* spp. as well as to reveal differences in the composition of the AMF communities of different clones. The use of DGGE, therefore, presents an opportunity to answer complex questions regarding the role that AMF play in supporting the production of biomass in SRIC systems.

The primer pair FLR3/FLR4 was successful in targeting AMF exclusively, and no other potentially coexistent fungi were detected. Moreover, the sequences obtained from the excised bands allowed the establishment of their phylogenetic association with AMF species that have already been described. Unlike other fungi, the spores of AMF possess multiple nuclei and a high interspecific variation can be found even between nuclei of the same spore (Rodriguez *et al.*

2004). These and other unique characteristics have been challenging for the design of suitable primers which are indispensable for the use of PCR-based molecular techniques (Schussler *et al.* 2001a). The primer pair FLR3/FLR4 targets the conserved region of the large ribosomal subunit which appears to be more informative for AMF than either the ITS or the small ribosomal subunit regions (Gollotte *et al.* 2004). However, it has been reported that multiple sequences of the ribosomal gene can be found in the same AMF species (Rodriguez *et al.* 2004), and in some cases the variability between the sequences has been found to be as high as 19% (Rodriguez *et al.* 2005). This could result in several bands being observed in the DGGE gel even if the DNA has been obtained from one single species. In other words, it could cause an overestimation of the AMF diversity. Therefore, for future works it is recommendable to complement the results obtained from the DNA extraction from roots, with a profile of DNA obtained from individual spores of some of the AMF species identified here.

The species identified in this study are ubiquitous throughout natural ecosystems as well as in agricultural soils. Within the phylum Glomeromycota, the genus *Glomus* is perhaps the most commonly found and broadly spread (Redecker and Raab 2006). Species belonging to this genus, including *G. claroideum*, *G. irregulare* (*G. intraradices*) (Stockinger *et al.* 2009) and *G. mosseae*, associate with a wide variety of plant species. For a long time it was thought that AMF were not host-specific since the same fungi could colonize the roots of different plant species. However, this view has been disputed by several studies (Bever *et al.* 2001) which demonstrate that in fact the origin and identity of both the fungus and the plant host determines to a great extent the outcome of such association. In the case of *S. repens*, for example, it was shown that cuttings obtained from sites with different nutrients availability respond differently to the inoculation of the same AMF (Van der Heijden and Kuyper 2001a). Furthermore, the fact that a clear group of non-described AMF (Glo-S) (Figure 5-4) was found in this study may indicate a co-dependency between the function of this group and the presence of specific *Salix* clones. Therefore, the possibility that the performance of one clone varies according to the AMF communities present in each site where it is planted should be considered.

Differences in the susceptibility of willow genotypes to fungal colonization have been previously reported (Puttsepp *et al.* 2004). Similarly, in this work it was evident that the seven clones studied differed in their associations with AMF. Moreover, some degree of specificity was suggested by these results. Firstly, it was noted that clones *SX61* and *SX64* did not associate

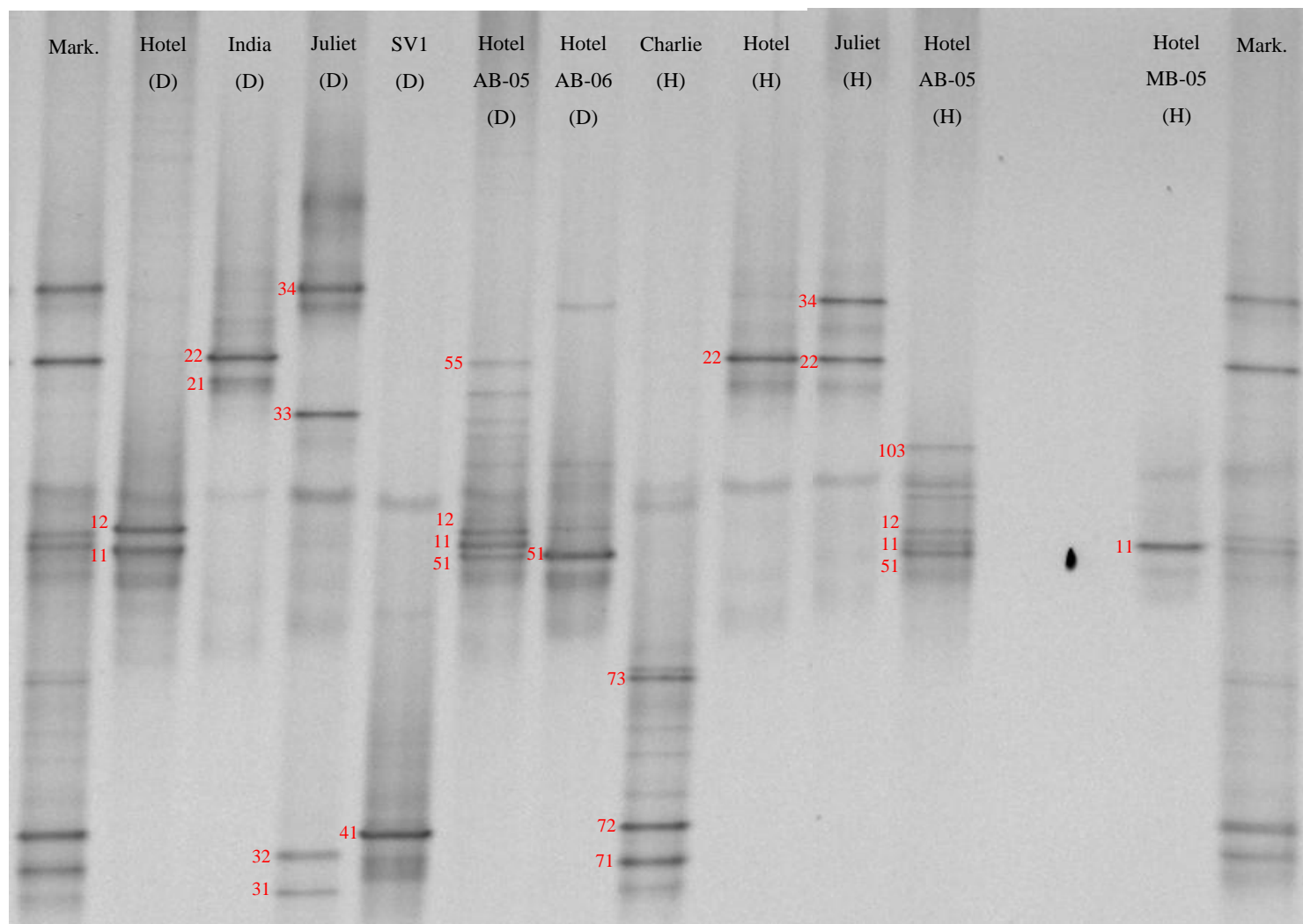


Figure 5-3. DGGE band profile of partial rDNA large subunit sequences from roots of healthy (*H*) and diseased (*D*) willows.

MB: Manitoba; *AB*: Alberta; 2005: 05, 2006: 06; Clones: *Charlie*, *Hotel*, *India*, *Juliet* and *SV1*; Mark.: marker.

Table 5-2. Sequence analyses of the bands excised from the gel after running a DGGE. The amplification was performed with mycorrhiza-specific primer pair FLR3/FLR4.

Band [§]	Most closely related sequence in the GenBank database	Description	Percent identity
73	AB280094.1	Uncultured glomeromycete	75%
31	DQ469095.1	Glomus claroideum clone 282.12	94%
41	DQ469095.1	Glomus claroideum clone 282.12	98%
72	DQ468802.1	Uncultured glomeromycete clone fe8	99%
71	DQ468802.1	Uncultured glomeromycete clone fe8	98%
12	EF554419.1	Uncultured fungus isolate qMpol38A	98%
51	EF554544.1	Uncultured fungus isolate qMpol3E	99%
103	DQ469111.1	Glomus cf. intraradices clone 488.5 [◇]	97%
11	AJ854604.1	Glomus sp. MUCL 43205	93%
21	FJ820960.1	Uncultured glomeromycete clone F5T	93%
22	FJ820960.1	Uncultured glomeromycete clone F5T	93%
32	FN643127.1	Uncultured glomeromycete	95%
34	EF554481.1	Uncultured fungus isolate	99%
55	GQ330816.1	Glomus mosseae isolate 79AU8	82%
33	EU380052.1	Uncultured glomeromycete	100%

[§]Band number according to Figure 5-3.

[◇]*G. irregulare* according to Stockinger (2009).

with AMF. These two clones are imported from Asia (Kopp 2001), and have previously demonstrated to stand apart of the other five clones regarding their association with other non-AMF root-living fungi (see Chapter 3). This appears to be very significant since both *SX61* and *SX64* have been reported to be highly productive and able to grow well under harsh conditions (Labrecque and Teodorescu 2005). On the other hand, the results for *Hotel* clearly indicated that the AMF communities associated with this clone share similarities and tend to cluster together irrespective of the health status or the province where it has been planted (Figure 5–5). Thus, the band identified with the number 11, which was placed in the cluster of *G. irregulare* (*G. intraradices*) (Figure 5-4), appeared to be associated with *Hotel* in all three provinces. However, in terms of diversity, *Hotel* plants cultivated in Manitoba presented a less complex AMF community compared to plants from Saskatchewan and Alberta (Figure 5–3). This could be the result of differential management practices of each site and remains an important factor to be explored in future works.

Differences in the composition of AMF communities were also observed between clones *SVI* and *India* (Figure 5–3) despite the fact that both are derived from the same parental species, *S. dasyclados*. However it was noticeable that for both clones only diseased plants appeared to be associated with AMF. In the case of the *SVI* clone the band was clustered in the group of *G. claroideum* (Figure 5-4) whereas the bands of *India* matched only with sequences of clone libraries but with no taxonomic names assigned. Although negative effects are rarely attributed to AMF, there are reports where the inoculation of AMF has resulted in the detriment of plant development. For example, Oliveira *et al.* (2006) reported negative effects of *G. claroideum* on the growth of *Salix atrocinerea*. The same fungus, however, induced an increase in biomass production on *Conyza bilbaoana*, which in natural ecosystems grows together with *S. atrocinerea*. Therefore, the possibility that *G. claroideum* happens to be beneficial for one clone and detrimental for another may be considered to explain the fact that it was found exclusively in diseased plants of *SVI* but in healthy plants of *Charlie* (Figure 5–3). In order to prove such hypothesis it would be necessary to compare the mycorrhizal dependency of both clones (Oliveira *et al.* 2006) and quantitatively determine the effect of *G. claroideum* on their biomass production capacity.

The results of the present work give no definitive evidence to support the involvement of AMF in the differential performance, in terms of biomass production, of the clones studied.

Nonetheless, in light of the new findings of the role of AMF in plant ecology (Bever *et al.* 2010), it is reasonable to think that the degree of compatibility between AMF and specific *Salix* clones may affect the ability of those clones to thrive and grow under SRIC. It has been well documented that the ability of an AMF species to colonize a plant does not imply benefits to the host (Johnson *et al.* 1997). Moreover, the mycorrhizal association is regarded as a continuum which, depending partially upon the environmental conditions, moves between being highly beneficial for both organisms and being detrimental for one them (Johnson *et al.* 1997). As it was stated above, it is important that future works consider the role of variables besides those included in this work. Variables such as the amount of native inoculum, the patchy distribution of AMF populations typical of agricultural land and the diversity of AMF may be studied in relation to the performance of specific willow clones grown under SRIC.

Finally, understanding the role of AMF communities in willow SRIC may constitute a fundamental step in the development of these systems in a sustainable and integrative way. The knowledge accumulated from previous attempts at mycorrhizal inoculation on poplars (Quoreshi *et al.* 2008), together with the information from new molecular techniques, and the possibility to monitor rapidly and accurately the communities of AMF, present an advantageous opportunity for the potential use of AMF in the development of biomass plantations. Some applications that may be considered are the inoculation of AMF to stimulate the establishment of the cuttings or the role that AMF play on the ability of willows to mobilize heavy-metals (Baum *et al.* 2006). Furthermore, a realistic evaluation of the nutritional and resistance benefits that willows may derive from the application of AMF could give an indication of the pertinence of using mycorrhizal inocula on SRIC. Addressing these questions is now possible thanks to the ability to accurately monitor AMF communities regardless of the size of the systems or the diversity of the fungal communities.

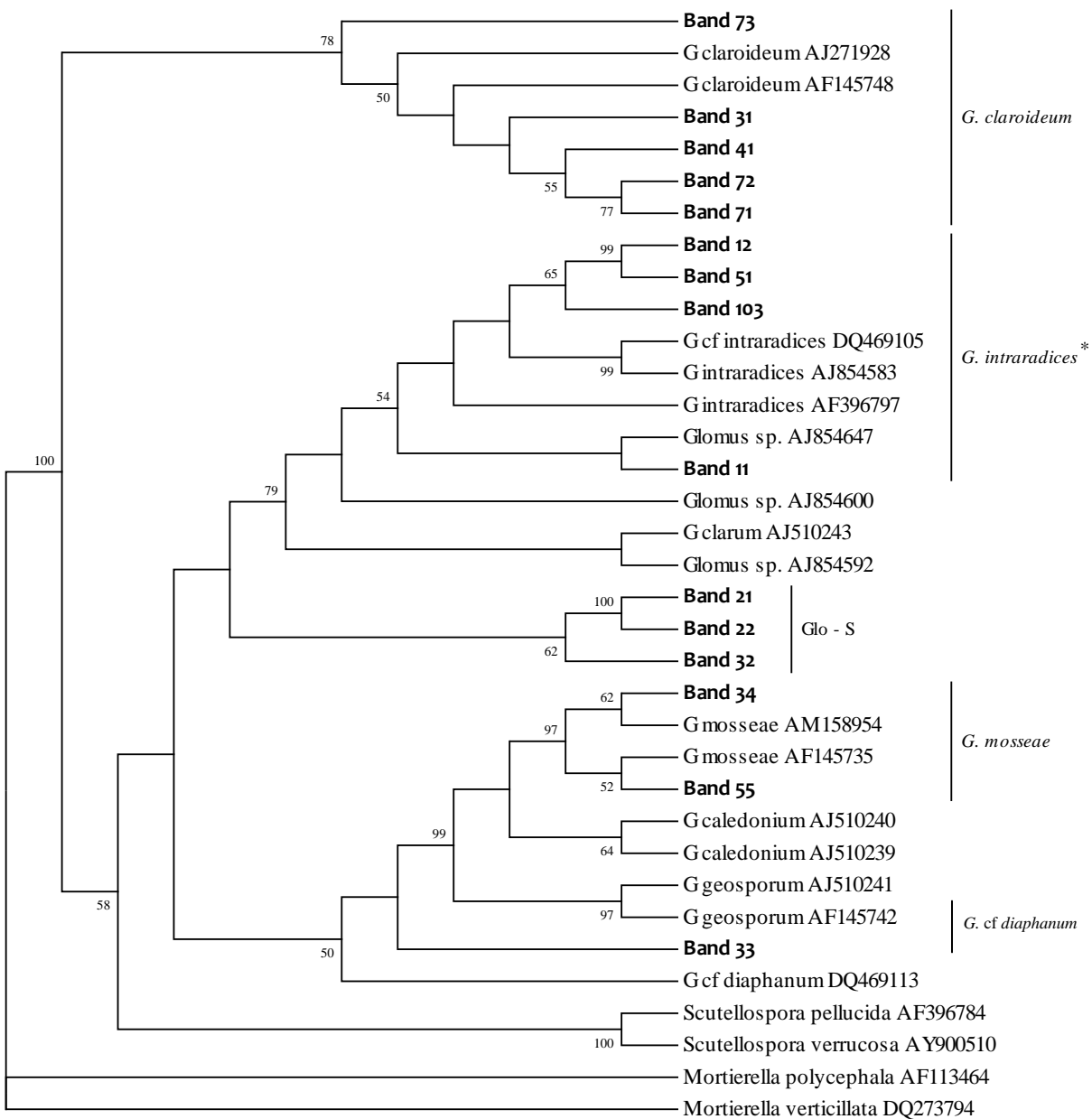


Figure 5-4. Phylogenetic analysis of AMF using the bands excised from the gel (in boldface) after running DGGE and sequences downloaded from the GenBank database. *Mortierella* spp. were used as the outer group. Numbers in the branches are bootstrap values from 100 iterations in a Maximum Likelihood analysis (only values higher than 50 are shown). The number next to the names of the sequences downloaded from GenBank corresponds to their accession numbers. * Probably *G. irregulare* according to Stockinger (2009).

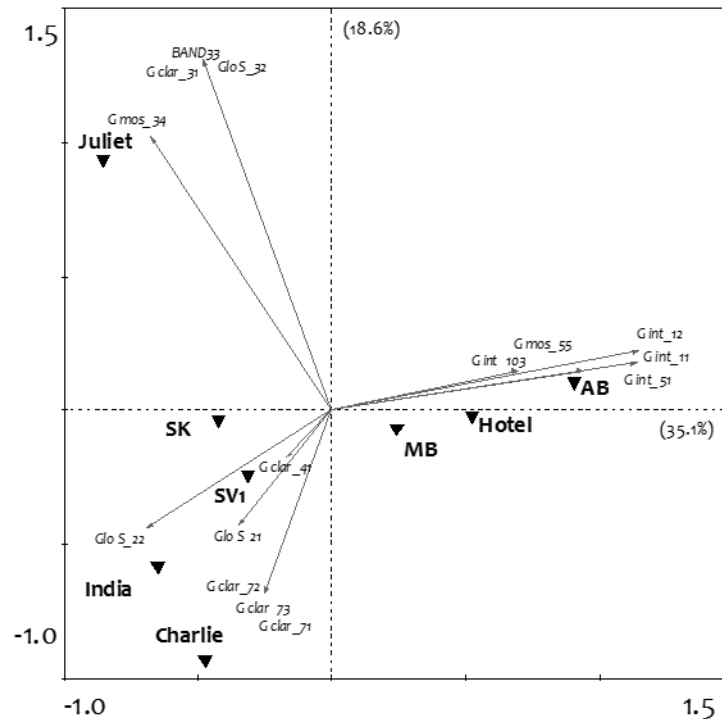


Figure 5-5. Principal correspondence analysis of AMF taxa, clone and province data. Names of AMF taxa correspond to the groups identified with the phylogenetic analysis (Figure 5–4) followed by the number of the band. The variability associated with each axis is presented inside the brackets. Legend of the species: G_{clar}: *Glomus claroideum*; G_{int}: *G. intraradices*; G_{mos}: *G. mosseae*; GloS: *Glo-S*; Uncult: Uncultured glomeromycete. Provinces: MB: Manitoba; SK: Saskatchewan; AB: Alberta. Willow clones: *Charlie*, *Hotel*, *India*, *Juliet* and *SV1*.

6. REFERENCES

- Abrahamson, L.P., Robison, D.J., Volk, T.A., White, E.H., Neuhauser, E.F., Benjamin, W.H., and Peterson, J.M. 1998. Sustainability and environmental issues associated with willow bioenergy development in New York (USA). *Biomass & Bioenergy* **15**(1): 17-22.
- Adams, P., De-Leij, F.A.A.M., and Lynch, J.M. 2007. *Trichoderma harzianum* Rifai 1295-22 mediates growth promotion of crack willow (*Salix fragilis*) saplings in both clean and metal-contaminated soil. *Microbial Ecology* **54**(2): 306-313.
- Agrios, G.N. 2005. Plant pathology. 5th ed. Elsevier Academic Press, Burlington, MA. 922 p.
- Akamatsu, H. 1999. Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. *Current Genetics* **35**(6): 647.
- Anderson, I.C., and Cairney, J.W.G. 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology* **6**(8): 769-779.
- Argus, G.W. 2007. *Salix* (Salicaceae) distribution maps and a synopsis of their classification in North America, North of Mexico. *Harvard Papers in Botany* **12**(2): 335-368.
- Arnebrant, K., Baath, E., and Soderstrom, B. 1990. Changes in microfungal community structure after fertilization of Scots Pine forest soil with ammonium-nitrate or urea. *Soil Biology & Biochemistry* **22**(3): 309-312.
- Atkins, S.D., and Clark, I.M. 2004. Fungal molecular diagnostics: A mini review. *Journal of Applied Genetics* **45**(1): 3-15.
- Babana, A.H., and Antoun, H. 2006. Effect of Tilemsi phosphate rock-solubilizing microorganisms on phosphorus uptake and yield of field-grown wheat (*Triticum aestivum* L.) in Mali. *Plant and Soil* **287**(1-2): 51-58.

- Ball, J., Carle, J., and Del Lungo, A. 2005. Contribution of poplars and willows to sustainable forestry and rural development. *Unasylva* **56**(221): 3-9.
- Baum, C., and Hryniewicz, K. 2006. Clonal and seasonal shifts in communities of saprotrophic microfungi and soil enzyme activities in the mycorrhizosphere of *Salix* spp. *Journal of Plant Nutrition and Soil Science* **169**(4): 481-487.
- Baum, C., Hryniewicz, K., Leinweber, P., and Meissner, R. 2006. Heavy-metal mobilization and uptake by mycorrhizal and nonmycorrhizal willows (*Salix x dasyclados*). *Journal of Plant Nutrition and Soil Science* **169**(4): 516-522.
- Baum, C., Leinweber, P., Weih, M., Lamersdorf, N., and Dimitriou, L. 2009. Effects of short rotation coppice with willows and poplar on soil ecology. *Landbauforschung Volkenrode* **59**(3): 183-196.
- Baum, C., Weih, M., Verwijst, T., and Makeschin, F. 2002. The effects of nitrogen fertilization and soil properties on mycorrhizal formation of *Salix viminalis*. *Forest Ecology and Management* **160**(1-3): 35-43.
- Beauchamp, V.B., Stromberg, J.C., and Stutz, J.C. 2006. Arbuscular mycorrhizal fungi associated with *Populus-Salix* stands in a semiarid riparian ecosystem. *New Phytologist* **170**(2): 369-380.
- Becerra, A.G., Nouhra, E.R., Silva, M.P., and McKay, D. 2009. Ectomycorrhizae, arbuscular mycorrhizae, and dark-septate fungi on *Salix humboldtiana* in two riparian populations from central Argentina. *Mycoscience* **50**(5): 343-352.
- Begley, D., McCracken, A.R., Dawson, W.M., and Watson, S. 2008. Interaction in short rotation coppice willow, *Salix viminalis* genotype mixtures. *Biomass and Bioenergy* **33**(2): 163-173.

Bever, J.D., Dickie, I.A., Facelli, E., Facelli, J.M., Klironomos, J., Moora, M., Rillig, M.C., Stock, W.D., Tibbett, M., and Zobel, M. 2010. Rooting theories of plant community ecology in microbial interactions. *Trends in Ecology and Evolution* **25**(8): 468-478.

Bever, J.D., Schultz, P.A., Pringle, A., and Morton, J.B. 2001. Arbuscular mycorrhizal fungi: More diverse than meets the eye, and the ecological tale of why. *Bioscience* **51**(11): 923.

Borneman, J., and Hartin, R.J. 2000. PCR primers that amplify fungal rRNA genes from environmental samples. *Applied and Environmental Microbiology* **66**(10): 4356-4360.

Bridge, P., and Spooner, B. 2001. Soil fungi: diversity and detection. *Plant and Soil* **232**(1-2): 147-154.

Environment Canada. Canada's National Climate Archive. Available from <http://climate.weatheroffice.gc.ca/> [accessed July 2010].

Chatli, A.S., Beri, V., and Sidhu, B.S. 2008. Isolation and characterisation of phosphate solubilising microorganisms from the cold desert habitat of *Salix alba* Linn. in trans Himalayan region of Himachal Pradesh. *Indian Journal of Microbiology* **48**(2): 267-273.

Chaverri, P., and Samuels, G.J. 2003. *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Studies in Mycology*(48): 1-116.

Christersson, L. 2008. Poplar plantations for paper and energy in the South of Sweden. *Biomass & Bioenergy* **32**(11): 997-1000.

Coutinho, T.A. 1998. *Eucalyptus* rust: a disease with the potential for serious international implications. *Plant Disease* **82**(7): 819-825.

De Cal, A., Garcia-Lepe, R., and Melgarejo, P. 2000. Induced resistance by *Penicillium oxalicum* against *Fusarium oxysporum* f. sp. *lycopersici*: Histological studies of infected and induced tomato stems. *Phytopathology* **90**(3): 260-268.

- De Lange, W.J., Wingfield, B.D., and Wingfield, M.J. 1998. A rapid, apple-based test for virulence in *Cryphonectria cubensis* isolates. *European Journal of Forest Pathology* **28**(6): 409-412.
- Desjardins, A.E. 2006. *Fusarium mycotoxins : chemistry, genetics and biology*. APS Press, St. Paul, Minn. 260 p.
- Dhillion, S.S. 1994. Ectomycorrhizae, arbuscular mycorrhizae, and *Rhizoctonia* sp. of alpine and boreal *Salix* spp. in Norway. *Arctic and Alpine Research* **26**(3): 304-307.
- Dhingra, O.D., and Sinclair, J.B. 1995. *Basic plant pathology methods*. 2nd ed. Lewis Publishers, Boca Raton. 434 p.
- Dimitriou, I., and Aronsson, P. 2005. Willows for energy and phytoremediation in Sweden. *Unasylva* **56**(221): 47-50.
- Dobranic, J.K., and Zak, J.C. 1999. A microtiter plate procedure for evaluating fungal functional diversity. *Mycologia* **91**(5): 756-765.
- Doran, J.W., and Zeiss, M.R. 2000. Soil health and sustainability: managing the biotic component of soil quality. *Applied Soil Ecology* **15**(1): 3-11.
- Douhan, G.W., Petersen, C., Bledsoe, C.S., and Rizzo, D.M. 2005. Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific amplification? *Mycorrhiza* **15**(5): 365-372.
- Fang, J.G., and Tsao, P.H. 1995. Efficacy of *Penicillium funiculosum* as a biological control agent against *Phytophthora* root rots of *Azalea* and *Citrus*. *Phytopathology* **85**(8): 871-878.
- Fernando, A.A., and Currah, R.S. 1996. A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. *Canadian Journal of Botany* **74**(7): 1071-1078.

- Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., and Rossi, P. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environmental Microbiology* **4**(11): 634-643.
- Fujimura, K.E., Egger, K.N., and Henry, G.H. 2008. The effect of experimental warming on the root-associated fungal community of *Salix arctica*. *ISME Journal* **2**(1): 105-114.
- Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes: Application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**(2): 113-118.
- Gebauer, G., and Taylor, A.F.S. 1999. N¹⁵ natural abundance in fruit bodies of different functional groups of fungi in relation to substrate utilization. *New Phytologist* **142**(1): 93-101.
- Gianinazzi, S. 2002. Mycorrhizal technology in agriculture: From genes to bioproducts. Birkhauser Verlag, Basel ; Boston. 296 p.
- Gollotte, A., van Tuinen, D., and Atkinson, D. 2004. Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. *Mycorrhiza* **14**(2): 111-117.
- Grizzle, H.W., and Zak, J.C. 2006. A microtiter plate procedure for evaluating fungal functional diversity on nitrogen substrates. *Mycologia* **98**(2): 353-363.
- Grunig, C.R., Queloz, V., Sieber, T.N., and Holdenrieder, O. 2008. Dark septate endophytes (DSE) of the *Phialocephala fortinii* s.l. - *Acephala applanata* species complex in tree roots: classification, population biology, and ecology. *Botany* **86**(12): 1355-1369.
- Hardle, W., and Simar, L. 2007. Correspondence Analysis. *In* Applied multivariate statistical analysis. Springer, Berlin ; New York. pp. xii, 458 p.
- Harman, G.E. 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**(2): 190-194.

- Hasegawa, M., Kishino, H., and Yano, T.A. 1985. Dating of the human ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**(2): 160-174.
- Helgason, T., Daniell, T.J., Husband, R., Fitter, A.H., and Young, J.P.W. 1998. Ploughing up the wood-wide web? *Nature* **394**(6692): 431-431.
- Hoppener-Ogawa, S., Leveau, J.H.J., Hundscheid, M.P.J., van Veen, J.A., and de Boer, W. 2009. Impact of *Collimonas* bacteria on community composition of soil fungi. *Environmental Microbiology* **11**(6): 1444-1452.
- Howard, A. 2006. *The soil and health : a study of organic agriculture*. University Press of Kentucky, Lexington. 307 p.
- Hryniewicz, K., Baum, C., and Leinweber, P. 2009. Mycorrhizal community structure, microbial biomass P and phosphatase activities under *Salix polaris* as influenced by nutrient availability. *European Journal of Soil Biology* **45**(2): 168-175.
- Hubbes, M. 1983. A review of the potential diseases of *Alnus* and *Salix* in energy plantations. International Energy Agency Programme Group B, Maple. 35 p.
- Hunter, T., Royle, D.J., and Arnold, G.M. 1996. Variation in the occurrence of rust (*Melampsora* spp) and other diseases and pests, in short-rotation coppice plantations of *Salix* in the British Isles. *Annals of Applied Biology* **129**(1): 1-12.
- Hutchison, L.J. 1999. Wood-inhabiting microfungi isolated from *Populus tremuloides* from Alberta and northeastern British Columbia. *Canadian Journal of Botany* **77**(6): 898-905.
- James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H.T., Rauhut, A., Reeb, V., Arnold, A.E., Amtoft, A., Stajich, J.E., Hosaka, K., Sung, G.H., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J.C., Wang, Z., Wilson, A.W., Schussler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W.,

White, M.M., Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A.Y., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E., Langer, G., Untereiner, W.A., Lucking, R., Budel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D.S., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W., and Vilgalys, R. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* **443**(7113): 818-822.

Johnson, N.C., Graham, J.H., and Smith, F.A. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* **135**(4): 575-586.

Jumpponen, A., and Trappe, J.M. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist* **140**(2): 295-310.

Kahle, P., Baum, C., and Boelcke, B. 2005. Effect of afforestation on soil properties and mycorrhizal formation. *Pedosphere* **15**(6): 754-760.

Khasa, P.D., Chakravarty, P., Robertson, A., Thomas, B.R., and Dancik, B.P. 2002. The mycorrhizal status of selected poplar clones introduced in Alberta. *Biomass & Bioenergy* **22**(2): 99-104.

Kinloch, B.B. 2003. White pine blister rust in North America: past and prognosis. *Phytopathology* **93**(8): 1044-1047.

Kisand, V., and Wikner, J. 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. *Journal of Microbiological Methods* **54**(2): 183-191.

Kopp, R.F. 2001. The development of improved willow clones for eastern North America. *The Forestry Chronicle* **77**(2): 287-292.

- Kowalchuk, G.A., De Souza, F.A., and Van Veen, J.A. 2002. Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. *Molecular Ecology* **11**(3): 571-581.
- Kuzovkina, Y.A., and Quigley, M.F. 2005. Willows beyond wetlands: uses of *Salix* L. species for environmental projects. *Water, air, and soil pollution* **162**: 183-204.
- Labrecque, M., and Teodorescu, T.I. 2003. High biomass yield achieved by *Salix* clones in SRIC following two 3-year coppice rotations on abandoned farmland in southern Quebec, Canada. *Biomass & Bioenergy* **25**(2): 135-146.
- Labrecque, M., and Teodorescu, T.I. 2005. Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). *Biomass & Bioenergy* **29**(1): 1-9.
- Labrecque, M., and Teodorescu, T.I. 2008. Important developments for short-rotation intensive culture of willow in different regions of eastern Canada *In* Short Rotation Crops International Conference, Minnesota, USA. p. 28.
- Lardizabal, K., Effertz, R., Levering, C., Mai, J., Pedroso, M.C., Jury, T., Aasen, E., Gruys, K., and Bennett, K. 2008. Expression of *Umbelopsis ramanniana* DGAT2A in seed increases oil in soybean. *Plant Physiology* **148**(1): 89-96.
- Leps, J., and Smilauer, P. 2003. Multivariate analysis of ecological data using CANOCO. Cambridge University Press, Cambridge, U.K. ; New York. 269 p.
- Liang, Z.B., Drijber, R.A., Lee, D.J., Dwiekat, I.M., Harris, S.D., and Wedin, D.A. 2008. A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil. *Soil Biology & Biochemistry* **40**(4): 956-966.

- Likar, M., and Regvar, M. 2009. Application of temporal temperature gradient gel electrophoresis for characterisation of fungal endophyte communities of *Salix caprea* L. in a heavy metal polluted soil. *Science of the Total Environment* **407**(24): 6179-6187.
- Lilleskov, E.A., Fahey, T.J., Horton, T.R., and Lovett, G.M. 2002. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology* **83**(1): 104-115.
- Longa, C.M.O., Savazzini, F., Tosi, S., Elad, Y., and Pertot, I. 2009. Evaluating the survival and environmental fate of the biocontrol agent *Trichoderma atroviride* SC1 in vineyards in northern Italy. *Journal of Applied Microbiology* **106**(5): 1549-1557.
- Ma, W.K., Siciliano, S.D., and Germida, J.J. 2005. A PCR-DGGE method for detecting arbuscular mycorrhizal fungi in cultivated soils. *Soil Biology & Biochemistry* **37**(9): 1589-1597.
- Magurran, A.E. 2004. *Measuring biological diversity*. Wiley-Blackwell Malden, MA. 256 p.
- Makeschin, F. 1994. Effects of energy forestry on soils. *Biomass & Bioenergy* **6**(1): 63-79.
- Mandyam, K., and Jumpponen, A. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology* **53**: 173-189.
- Mavragani, D.C. 2008. Molecular and ecological studies of fungal biodiversity on durum wheat grown in rotation with pulses and canola Department of Applied Microbiology and Food Science. University of Saskatchewan Saskatoon. p. 147.
- Milne, I. 2004. TOPALi: software for automatic identification of recombinant sequences within DNA multiple alignments. *Bioinformatics* **20**(11): 1806-1807.
- Molina, R., Massicotte, H., and Trappe, J. 1992. Specificity phenomena in mycorrhizal symbioses: Community ecological consequences and practical implications. *In* *Mycorrhizal*

functioning: an integrative plant-fungal process. *Edited by Michael F. Allen*. Chapman & Hall, New York ; London. pp. 357-423.

Muhlmann, O., and Peintner, U. 2008. Mycobionts of *Salix herbacea* on a glacier forefront in the Austrian Alps. *Mycorrhiza* **18**(4): 171-180.

Mulder, C., Breure, A.M., and Joosten, J.H.J. 2003. Fungal functional diversity inferred along Ellenberg's abiotic gradients: Palynological evidence from different soil microbiota. *Grana* **42**(1): 55-64.

Muyzer, G., Dewaal, E.C., and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal RNA. *Applied and Environmental Microbiology* **59**(3): 695-700.

Muyzer, G., and Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **73**(1): 127-141.

Nakatsu, C.H. 2007. Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Science Society of America Journal* **71**(2): 562-571.

Obanor, F., Erginbas-Orakci, G., Tunali, B., Nicol, J.M., and Chakraborty, S. 2010. *Fusarium culmorum* is a single phylogenetic species based on multilocus sequence analysis. *Fungal Biology* **114**(9): 753-765.

Oliveira, R.S., Castro, P.M.L., Dodd, J.C., and Vosatka, M. 2006. Different native arbuscular mycorrhizal fungi influence the coexistence of two plant species in a highly alkaline anthropogenic sediment. *Plant and Soil* **287**(1-2): 209-221.

Paradi, I., and Baar, J. 2006. Mycorrhizal fungal diversity in willow forests of different age along the river Waal, The Netherlands. *Forest Ecology and Management* **237**(1-3): 366-372.

Pei, M.H., Royle, D.J., and Hunter, T. 1996. Pathogenic specialization in *Melampsora epitea* var *epitea* on *Salix*. Plant Pathology **45**(4): 679-690.

Puttsepp, U., Rosling, A., and Taylor, A.F.S. 2004. Ectomycorrhizal fungal communities associated with *Salix viminalis* L. and *S. dasyclados* Wimm. clones in a short-rotation forestry plantation. Forest Ecology and Management **196**(2-3): 413-424.

Quaggiotti, S., Barcaccia, G., Schiavon, M., Nicole, S., Galla, G., Rossignolo, V., Soattin, M., and Malagolia, M. 2007. Phytoremediation of chromium using *Salix* species: Cloning ESTs and candidate genes involved in the Cr response. Gene **402**(1-2): 68-80.

Quoreshi, A.M., Piche, Y., and Khasa, D.P. 2008. Field performance of conifer and hardwood species 5 years after nursery inoculation in the Canadian Prairie Provinces. New Forests **35**(3): 235-253.

Redecker, D., and Raab, P. 2006. Phylogeny of the Glomeromycota (arbuscular mycorrhizal fungi): Recent developments and new gene markers. Mycologia **98**(6): 885-895.

Rodriguez, A., Clapp, J.P., and Dodd, J.C. 2004. Ribosomal RNA gene sequence diversity in arbuscular mycorrhizal fungi (Glomeromycota). Journal of Ecology **92**(6): 986-989.

Rodriguez, A., Clapp, J.P., Robinson, L., and Dodd, J.C. 2005. Studies on the diversity of the distinct phylogenetic lineage encompassing *Glomus claroideum* and *Glomus etunicatum*. Mycorrhiza **15**(1): 33-46.

Ronnberg-Wastljung, A.C., Samils, B., Tsarouhas, V., and Gullberg, U. 2008. Resistance to *Melampsora larici-epitea* leaf rust in *Salix*: Analyses of quantitative trait loci. Journal of Applied Genetics **49**(4): 321-331.

Rooney, D.C., Killham, K., Bending, G.D., Baggs, E., Weih, M., and Hodge, A. 2009. Mycorrhizas and biomass crops: Opportunities for future sustainable development. Trends in Plant Science **14**(10): 542-549.

Rossman, A.Y. 2007. A review of the phylogeny and biology of the Diaporthales. *Mycoscience* **48**(3): 135-144.

Royle, D.J., and Ostry, M.E. 1995. Disease and pest control in the bioenergy crops poplar and willow. *Biomass & Bioenergy* **9**(1-5): 69-79.

Sage. 1999. Weed competition in willow coppice crops: the cause and extent of yield losses. *Weed Research* **39**(5): 399-411.

Sanders, I.R., Alt, M., Groppe, K., Boller, T., and Wiemken, A. 1995. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales - Application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. *New Phytologist* **130**(3): 419-427.

Sasaki, A., Fujiyoshi, M., Shidara, S., and Nakatsubo, T. 2001. Effects of nutrients and arbuscular mycorrhizal colonization on the growth of *Salix gracilistyla* seedlings in a nutrient-poor fluvial bar. *Ecological Research* **16**(1): 165-172.

Schroers, H.J. 2001. A monograph of *Bionectria* (Ascomycota, Hypocreales, Bionectriaceae) and its *Clonostachys* anamorphs. *Studies in Mycology* **46**: 1-214.

Schussler, A., Gehrig, H., Schwarzott, D., and Walker, C. 2001a. Analysis of partial Glomales SSU rRNA gene sequences: Implications for primer design and phylogeny. *Mycological Research* **105**(1): 5-15.

Schussler, A., Schwarzott, D., and Walker, C. 2001b. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research* **105**(12): 1413-1421.

Slapokas, T., and Granhall, U. 1991. Decomposition of willow-leaf litter in a short rotation forest in relation to fungal colonization and palatability for earthworms. *Biology and Fertility of Soils* **10**(4): 241-248.

- Smaliukas, D., and Noreika, R. 2005. Morphobiological characteristics and evaluation of productivity of *Salix* L. clones selected for short-rotation coppice. *Biologija* **3**: 36-40.
- Smart, L.B., Volk, T.A., Lin, J., Kopp, R.F., Phillips, I.S., Cameron, K.D., White, E.H., and Abrahamson, L.P. 2005. Genetic improvement of shrub willow (*Salix* spp.) crops for bioenergy and environmental applications in the United States. *Unasylva* **56**(221): 51-55.
- Smith, S.E., and Gianinazzi-Pearson, V. 1990. Phosphate uptake and arbuscular activity in mycorrhizal *Allium cepa* L.: Effects of photon irradiance and phosphate nutrition. *Australian Journal of Plant Physiology* **17**(2): 177-188.
- Spiers, A.G. 1998. *Melampsora* and *Marssonina* pathogens of poplars and willows in New Zealand. *European Journal of Forest Pathology* **28**(4): 233-240.
- Statistics Canada. Agriculture Division. 2004. Canadian agriculture at a glance. Minister of Industry, Ottawa. 341 p.
- Stockinger, H., Walker, C., and Schussler, A. 2009. 'Glomus intraradices DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*. *New Phytologist* **183**(4): 1176-1187.
- Summerbell, R.C. 2005. From Lamarckian fertilizers to fungal castles: recapturing the pre-1985 literature on endophytic and saprotrophic fungi associated with ectomycorrhizal root systems. *Studies in Mycology* **53**: 191-256.
- Sylvia, D.M. 2005. Principles and applications of soil microbiology. 2nd ed. Pearson Prentice Hall, Upper Saddle River, N.J. 640 p.
- Tamura, K. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**(8): 1596-1599.

ter Braak, C., and Smilauer, P. 2002. CANOCO, Reference manual and CanoDraw for Windows user's guide: Software for canonical community ordination, (version 4.5). Microcomputer Power, Ithaca. 500 p.

Trillas, M.I., and Segarra, G. 2009. Interactions between nonpathogenic fungi and plants. *Advances in Botanical Research* **51**(C): 321-359.

Trouvelot, S., van Tuinen, D., Hijri, M., and Gianinazzi-Pearson, V. 1999. Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence in situ hybridization. *Mycorrhiza* **8**(4): 203-206.

Trowbridge, J., and Jumpponen, A. 2004. Fungal colonization of shrub willow roots at the forefront of a receding glacier. *Mycorrhiza* **14**(5): 283-293.

Vainio, E.J., and Hantula, J. 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research* **104**: 927-936.

Van Bruggen, A.H.C., and Semenov, A.M. 2000. In search of biological indicators for soil health and disease suppression. *Applied Soil Ecology* **15**(1): 13-24.

Van der Heijden, E.W. 2001. Differential benefits of arbuscular mycorrhizal and ectomycorrhizal infection of *Salix repens*. *Mycorrhiza* **10**(4): 185-193.

Van der Heijden, E.W., and Kuyper, T.W. 2001a. Does origin of mycorrhizal fungus or mycorrhizal plant influence effectiveness of the mycorrhizal symbiosis? *Plant and Soil* **230**(2): 161-174.

Van der Heijden, E.W., and Kuyper, T.W. 2001b. Laboratory experiments imply the conditionality of mycorrhizal benefits for *Salix repens*: role of pH and nitrogen to phosphorus ratios. *Plant and Soil* **228**(2): 275-290.

- Van der Heijden, E.W., Vries, F.W., and Kuyper, T.W. 1999. Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. I. Above-ground and below-ground views of ectomycorrhizal fungi in relation to soil chemistry. *Canadian Journal of Botany* **77**(12): 1821-1832.
- Van der Heijden, M.G.A., and Sanders, I.R. 2002. *Mycorrhizal ecology*. Springer, Berlin ; New York. 469 p.
- Van der Lelie, D., Taghavi, S., Monchy, S., Schwender, J., Miller, L., Ferrieri, R., Rogers, A., Wu, X., Zhu, W., Weyens, N., Vangronsveld, J., and Newman, L. 2009. Poplar and its bacterial endophytes: coexistence and harmony. *Critical Reviews in Plant Sciences* **28**(5): 346-358.
- Van Elsas, J.D. 2002. Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. *Biodegradation* **13**(1): 29-40.
- Van Elsas, J.D., Duarte, G.F., Keijzer-Wolters, A., and Smit, E. 2000. Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. *Journal of Microbiological Methods* **43**(2): 133-151.
- Van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., and Gianinazzi-Pearson, V. 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Molecular Ecology* **7**(7): 879-887.
- Visentin, I., Tamietti, G., Valentino, D., Portis, E., Karlovsky, P., Moretti, A., and Cardinale, F. 2009. The ITS region as a taxonomic discriminator between *Fusarium verticillioides* and *Fusarium proliferatum*. *Mycological Research* **113**: 1137-1145.
- Volk, T.A., Abrahamson, L.P., Nowak, C.A., Smart, L.B., Tharakan, P.J., and White, E.H. 2006. The development of short-rotation willow in the northeastern United States for bioenergy and bioproducts, agroforestry and phytoremediation. *Biomass & Bioenergy* **30**(8-9): 715-727.

- Volk, T.A., Verwijst, T., Tharakan, P.J., Abrahamson, L.P., and White, E.H. 2004. Growing fuel: a sustainability assessment of willow biomass crops. *Frontiers in Ecology and the Environment* **2**(8): 411-418.
- Vujanovic, V., Hamel, C., Yergeau, E., and St-Arnaud, M. 2006. Biodiversity and biogeography of *Fusarium* species from northeastern North American asparagus fields based on microbiological and molecular approaches. *Microbial Ecology* **51**(2): 242-255.
- Vujanovic, V., Hamelin, R.C., Bernier, L., Vujanovic, G., and St-Arnaud, M. 2007. Fungal diversity, dominance, and community structure in the rhizosphere of clonal *Picea mariana* plants throughout nursery production chronosequences. *Microbial Ecology* **54**(4): 672-684.
- Vujanovic, V., and Labrecque, M. 2002. Biodiversity of pathogenic mycobiota in *Salix* bioenergy plantations, Québec. *Canadian Plant Disease Survey* **82**: 138-139.
- Vujanovic, V., and Labrecque, M. 2008. Potentially pathogenic and biocontrol Ascomycota associated with green wall structures of basket willow (*Salix viminalis* L.) revealed by phenotypic characters and ITS phylogeny. *Biocontrol* **53**(2): 413-426.
- Werner, A., Zadworny, M., and Idzikowska, K. 2002. Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* grown in vitro. *Mycorrhiza* **12**(3): 139-145.
- White, T.J., Bruns, T.D., Lee, S.B., and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols - A guide to methods and applications. *Edited by* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White. Academic Press, New York. pp. 315-322.
- Woolhouse, M.E.J. 2005. Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in Ecology & Evolution* **20**(5): 238-244.

Wright, L. 2006. Worldwide commercial development of bioenergy with a focus on energy crop-based projects. *Biomass & Bioenergy* **30**(8-9): 706-714.

Yergeau, E., Filion, M., Vujanovic, V., and St-Arnaud, M. 2005. A PCR-denaturing gradient gel electrophoresis approach to assess *Fusarium* diversity in asparagus. *Journal of Microbiological Methods* **60**(2): 143-154.

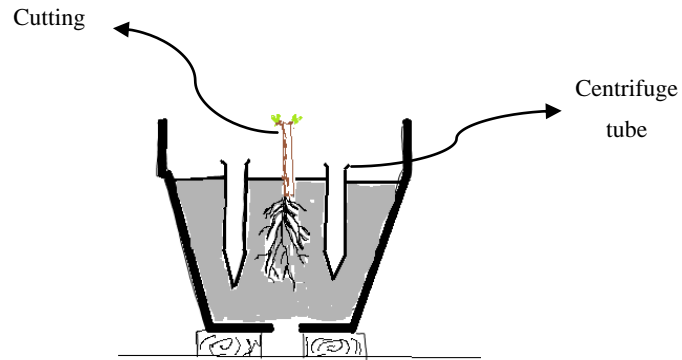
Yergeau, E., Vujanovic, V., and St-Arnaud, M. 2006. Changes in communities of *Fusarium* and arbuscular mycorrhizal fungi as related to different asparagus cultural factors. *Microbial Ecology* **52**(1): 104-113.

Zak, J.C., and Visser, S. 1996. An appraisal of soil fungal biodiversity: The crossroads between taxonomic and functional biodiversity. *Biodiversity and Conservation* **5**(2): 169-183.

APPENDIX

INOCULATION OF PATHOGENIC FUNGI WITHOUT DISTURBING THE ROOT SYSTEM

a



b



Figure A-1. Setup used for the inoculation of potentially pathogenic fungi in cuttings of *Hotel* clone
a) Schematic diagram. Adapted from Dhingra and Sinclair (1998) **b)** Cutting transplanted after the inoculation with the potentially beneficial fungi. Forty-eight hours later the centrifuge tubes were removed and the wells were filled with the suspension of the potentially pathogenic fungi.